**Determining the Population Structure and Avirulence Gene Repertoire of the Rice Blast Fungus *Magnaporthe oryzae* in Kenya by Comparative Genome Analysis**

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I certify that all material in the thesis which is not my own work has been identified and that
no material has previously been submitted and approved for the award of a degree by this or any other University.

**David Mwongera**

**Abstract**

Rice blast disease is caused by the ascomycete fungus *Magnaporthe oryzae* and is of economic importance worldwide, due to its wide geographical distribution and the severe yield losses it causes on cultivated rice. Understanding the population structure of *M. oryzae* is key to sustainable management of blast disease. In this study, a total of 290 *M. oryzae* isolates were collected from rice growing regions in Kenya including Central Kenya (Mwea irrigation scheme), Western Kenya (Ahero and Maugo irrigation schemes in Ahero and Homa-Bay respectively) and Coastal Kenya (Kwale). Initially, I undertook genotyping of a subset of Kenyan isolates by DNA sequence analysis of the internal transcribed spacer regions (ITS 1 and ITS 2) of the rRNA-encoding gene unit and by DNA fingerprinting using the Pot2 repetitive DNA element. Phylogenetic analyses based on ITS sequences clustered together isolates from Western and Coastal Kenya which were distinct from Central Kenya isolates. Cluster analysis based on 80% DNA fingerprint similarity, identified five clonal lineages designated KL1, KL2, KL3, KL4 and KL5 with most isolates belonging to lineages KL2, KL3, KL4. The clustering of isolates was region specific with Western and Coastal isolates closely related to each other and distinct from Central Kenya isolates. Distribution of mating type gene loci (*MAT1.1* and *MAT1.2*) was determined using mating type gene specific primers. My results indicate that *MAT1.1* is the predominant mating type and is distributed in all the rice growing regions of Kenya. *MAT1.2* isolates were identified only in Coastal Kenya. I further undertook high throughput next-generation DNA sequencing of the genomes of 27 *M. oryzae* isolates from sub-Saharan Africa (SSA), including Kenya, Uganda, Tanzania, Benin, Togo, Nigeria and Burkina Faso and compared them to other sequenced strains from China, India, USA, Philippines, Thailand, Korea, Japan, France and French Guiana.

Single nucleotide polymorphisms (SNPs) indicated that majority of East African isolates of *M. oryzae* clustered separately from West African isolates. African isolates clustered with isolates from India and China, indicating that rice blast in SSA may have originated from Asia.

Pathotype analysis of Kenyan isolates was undertaken using a set of monogenic differential rice varieties, collectively harbouring 24 disease resistance genes. Rice blast resistance gene *Pi-z*5 conferred resistance to all the isolates tested. Other resistance genes that conferred resistance to majority of isolates tested include *Pi*-9, *Pi*-12*(t), Pi-ta, Pi-ta2* and *Pi-z*. These resistance genes are suitable candidates for introgressing into commercially grown varieties in Kenya in combinations. I also investigated the population of *M.* oryzae isolates to identify cognate avirulence gene loci, including novel genes not yet reported. Finally, I evaluated rice varieties grown in Kenya for resistance to indigenous rice blast isolates under laboratory conditions. Rice variety Basmati 370 was susceptible to rice blast with varieties IR2793-80-1, BW 196, NERICA 1, NERICA 4, NERICA 10, and NERICA 11 showing some disease resistance. Varieties ITA 310 and Duorado Precoce were moderately tolerant to rice blast. This information is being used to develop a durable blast resistance strategy in sub-Saharan Africa.

**Table of contents**

**List of Figures**

**List of Tables**

## List of appendices

**List of abbreviations**

| | |
|---|---|
| ANOVA | analysis of variance |
| bp | base pair |
| CM | complete medium |
| CTAB | hexadecyltrimethylammonium bromide |
| DFID | department for International development, UK |
| DNA | deoxyribonucleic acid |
| dNTP | 2'-deoxyribonucleoside 5' –triphosphate |
| EDTA | disodium ethylene diamine tetraacetic acid |
| EIHM | extra-invasive hyphal membrane |
| Fig | Figure |
| g | grams |
| g $L^{-1}$ | grams per litre |
| IH | invasive hypha |
| ITS1 | Internal Transcribed Spacer 1 |
| ITS2 | Internal Transcribed Spacer 2 |
| KALRO | Kenya Agricultural and Livestock Research Organisation |
| km | kilometre |
| $L^{-1}$ | per litre |
| LINE | Long Interspersed Nuclear Element |
| LTR | Long Terminal Repeat |
| MAT | mating type |
| m | metre |
| mg $l^{-1}$ | milligrams per litre |
| mg $ml^{-1}$ | milligrams per millitre |
| MGR | magnaporthe grisea repeat |
| min | minute |
| ml | millilitre |
| NaCl | sodium chloride |
| ng $\mu l^{-1}$ | nanogram per microlitre |
| PCR | polymerase chain reaction |

| | |
|---|---|
| pH | $\log_{10}$ hydrogen concentration |
| rRNA | ribosomal RNA |
| SINE | short Interspersed Nuclear Element |
| TBE | tris Borate-EDTA |
| Tris | tris (hyroxymeththyl) aminomethane |
| UK | United Kingdom |
| UPGMA | unweighted pairgroup method with arithmetic averages |
| w/v | weight per volume |
| WA | water agar |
| $^{O}$C | degrees Celsius |
| % | percent |

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## Dedication

This thesis is dedicated to my wife, Elizabeth Mwongera, and Children, Ivana and Lance for their immense patience during my long absence from home.

# 1 Chapter 1: General Introduction

## 1.1 Rice production in sub Saharan Africa

In the last two decades, there has been a very significant increase in annual consumption of rice in sub-Saharan Africa (SSA). This has been caused by a shift in consumer preferences, increasing urbanization and a rapid increase in the human population (Onyango, 2014). Annual rice production in SSA is estimated at 12.5 million metric tonnes (MT), compared with annual consumption of 24.3 million MT. Insufficient rice production in the region therefore affects 20 million people who depend on rice as their staple food. This necessitates importation of large quantities of rice amounting to 40% of total consumption. In the year 2009, for example, 9.68 million MT valued at USD 5 billion were imported into SSA (Onyango, 2014). This exerts considerable pressure on the already fragile economies of these countries.

### 1.1.1 Rice production in Kenya

Agriculture plays a critical role in the economic development of Kenya and economic growth is directly correlated to performance of the agriculture sector. Agriculture is the backbone of Kenya's economy and directly contributes 30% to the annual Gross Domestic Product and a further 27% indirectly which are valued at USD 4.5 billion and USD 5.1 billion, respectively. Furthermore, agriculture in Kenya is dominated by smallholder farmers and contributes up to 60% of the formal waged employment in both the private and public sectors (Kenya National Bureau of Statistics, 2016; Muma, 2016).

Rice was introduced into Kenya from Asia in 1907 and is now the third most important cereal crop in Kenya, after maize and wheat. Estimates from the Kenyan Ministry of Agriculture (MoA, 2008) indicate that rice is grown by about 300,000 smallholder farmers, both as a commercial crop and as a domestic food

crop. Production is estimated at 45,000-80,000 metric tonnes (MT) per annum with irrigated schemes and rain-fed conditions accounting for 80% and 20% of rice production, respectively. Most rain-fed rice is grown in Coastal Kenya in Kwale and Kilifi counties.

Rain-fed rice production is carried out in 5 irrigation schemes under the management of the National Irrigation Board. Mwea irrigation scheme, started in 1956, is the largest and is located in Kirinyaga county in the central part of Kenya. The scheme covers an area of 12,282 hectares (ha) with about 6,475 ha developed for irrigation, and is divided into five sections: Mwea covering (1,300 ha), Tebere (1,400 ha), Thiba (1,200 ha), Karaba 1,100 (ha) and Wamumu (1,200 ha). Each section is divided into units of about 100 ha with a total of 59 units. The scheme supports 3,400 smallholder households and accounts for 80% of the rice grown in Kenya. The Mwea irrigation scheme is known for producing the aromatic Basmati rice, which accounts for 80% of rice grown in the scheme (USAID 2010; Mati *et al,*. 2011; Kihoro *et al.*, 2013; Njeru *et al*., 2016). Three irrigation schemes, Ahero, West Kano and Bunyala, are located in western Kenya. Ahero irrigation scheme is located in Kisumu county and has an area of 840 ha. The scheme was initiated in 1969 and supports 520 smallholder farmers. West Kano irrigation scheme was established in 1975 and is located on the shores of Lake Victoria in Kisumu county. The scheme occupies 900 ha and supports approximately 550 farmers. Ahero and West Kano draw water for irrigation from River Nyando and Lake Victoria respectively. Bunyala irrigation scheme was established in 1968 and is located in Busia county, where it occupies 500 ha and serves about 300 farmers. The scheme receives its water from river Nzoia (Njeru *et al*., 2016). In addition to the smallholder farmers within the NIB irrigation schemes, irrigated rice is also grown by smallholder farmers outside the NIB irrigation schemes.

The main rice varieties grown in Kenya include Basmati 370, Basmati 217, BW 196, Duarado Precoce, ITA 310, IR2793-80-1, NERICA 1, NERICA 4, NERICA 10 and NERICA 11. The main characteristics of these varieties are described in detail in Chapter 4.

Importantly, rice production in Kenya falls short of the national requirement which is estimated to be 300,000 MT per annum, with consumption growing at a rate of 12% per annum. Compared with other cereal crops, this is the highest growth rate in consumption, with maize and wheat having growth rates of 1% and 4%, respectively (MoA, 2008). The shortfall in rice production is met by importation of rice from Pakistan, which accounts for 74% of imports, Vietnam accounting for 9%, with Thailand, India and Egypt each accounting for 4% of imports (MAFAP, 2013).

Smallholder rice yields average about 2.7 MT/ha and 4.7 MT/ha for non-irrigated and irrigated rice production systems, respectively, which is relatively low compared to potential yields of 8.6-10 MT/ha achieved within private large scale farms in the region (USAID, 2010). Low productivity has been attributed to abiotic, biotic and socio-economic factors (GoK, 2011). Drought has been a major challenge for upland rice farming systems. For example, in the drought period of 2008-2011, depressed rainfall was reported in most parts of the country with reductions ranging from 60-80%, compared to the long-term annual average, causing losses in rice production of more than 38,900 tons with a value of Ksh million 1828 (GoK, 2011). Moreover, even under irrigated farming systems, declining water resources due to global warming are already becoming a reality in the region (GoK, 2013).

Rice blast disease is the most important biotic factor affecting rice production in Kenya. It is found in all rice growing regions causing significant yield losses of 52% (Kihoro *et al*., 2013).

## 1.2 Rice blast disease

Rice blast disease is a serious disease of rice affecting rice worldwide with annual yield losses of between 10-30% attributable to the disease (Talbot, 2003). Rice blast disease is caused by the filamentous ascomycete fungus *Magnaporthe oryzae*. Couch (Couch and Kohn, 2002) (synonym of *Pyricularia oryzae*) (Zhang *et al.*, 2016) (previously named *Magnaporthe grisea* Sacc. (Talbot & Wilson, 2009). According to Zhang *et al.* (2016), the generic name *Pyricularia* was first used in 1880, based on asexual cultures of *P. grisea* collected from crabgrass (*Digitaria* spp). In 1892, Cavara assigned the name *P. oryzae* to rice-infecting isolates. *Pyricularia* and *Magnaporthe* are currently both widely used generic names for the rice blast fungus, with *Magnaporthe* being the most commonly used in all contemporary literature regarding the disease. Zhang *et al.* (2016) recommends the name *Pyricularia* as the correct name for the rice blast fungus because it corresponds the ecological and evolutionary features of the fungus, but they also recommend *Magnaporthe* as a synonym. Phylogenetic analysis based on a multilocus gene genealogy indicates that *Magnaporthe* spp. isolates cluster into two distinct clades. Those isolates that infect crabgrass cluster separately from those that infect rice, millets and grasses. Isolates infecting rice, millet and grasses have been re-named *M. oryzae*, while those infecting crabgrass retained the name *M. grisea* (Couch & Kohn, 2002). Rice infecting-populations of *M. oryzae* originated as a result of single host shift from *Setaria* millet to rice during the rice domestication era (Couch *et al*., 2005).

### 1.2.1 Symptoms

Rice blast symptoms form on leaves, nodes, panicles and grains (Mew *et al.,* 2016; Lanoiselet *et al.,* 2015). On rice leaves, disease lesions begin as small water-soaked whitish, greyish, or bluish spots. As the disease progresses, the lesions become elliptical and spindle-shaped, tapering at each end. The centre of the lesion is usually greyish or whitish, with a brown or reddish-brown margin (Figure 1-1) on susceptible rice varieties, fully developed disease lesions may reach 1–1.5 x 0.3–0.5 cm in length whilst on resistant genotypes the spots fail to enlarge and remain as minute, pin-sized brown specks. In susceptible rice varieties and under favourable conditions, disease lesions may coalesce killing the entire plant. The shape and colour of lesions varies depending on host genotype, environmental conditions and the developmental stage of the lesion. Lesions may also appear at the junction of the leaf blade and leaf sheath, and may kill the entire leaf. When areas near the panicle base are attacked, this results in neck rot, whereby blast symptoms start at the base of the panicle leading to necrosis of the entire panicle. Infected panicles are white and unfilled and may be confused with stem-borer attack caused by the African white rice stem borer (*Maliarpha separatella*). The fungus also infects the nodes of the plant in which case the nodes appear black-brown often occurring in a banded pattern and result in death of all plant parts above the node. This leads to significant disease losses of greater than 80% (Wilson and Talbot, 2009).

### 1.2.2 Host range and specificity

*M. oryzae* species are capable of causing blast disease in more than 50 monocot plant species, including food crops such as wheat, barley, rice and millets. Additionally, *M. oryzae* also infects wild grass hosts such as *Digitaria sanguinalis*, *Setaria viridis*, and *Eleusine indica* (Mew *et al.,* 2016). In addition, *M.*

*oryzae* isolates comprise host-specific sub-populations (pathotypes), based on the host they infect and include the *Oryza* pathotype (MoO) specific to rice, the *Triticum* pathotype (MoT) specific to wheat, the *Eleusine* pathotype (MoE) specific to finger millet, *Setaria* pathotype (MoS) specific to foxtail millet and the *Lolium* pathotype (MoL) specific to turf grasses (Couch *et al.*, 2005; Kato *et al.,* 2000). Recently, comparative genome analysis of host-specific forms of *M. oryzae* has been associated with gain and loss of genes linked to transposable elements (Yoshida *et al*., 2016).

### 1.2.3 Favourable environmental conditions

In the tropics, the environmental conditions that occur during the night are the most important factors contributing to occurrence of rice blast. Free water is required for all processes involved in infection by *M. oryzae* including spore production, release, germination and infection (Ou, 1980). In the tropics, night time temperatures are optimal for disease development and night time dew provides the required water for spore germination rice infection. The number of lesions produced is dependent on the duration of the dew (Ou, 1980). Upland rice is particularly prone to rice blast and this is thought to be due to a longer duration of dew experienced in these regions. In the tropics, lowland rice fields flooded with water have shorter dew periods than upland rice. This is because during the day the water is heated to temperatures up to 42$^{o}$C, and during the night the heat is released creating a micro-climate that delays dew formation (Ou, 1980).

Figure 1-1. Leaf blast symptoms on susceptible rice cultivar in Hunan province in China. Disease lesions enlarge up to 1 cm in length (as shown) on older rice leaves and can sporulate for up to two weeks, leading to rapid inoculum build up. Photograph: Nick Talbot

### 1.2.4 The biology and infection cycle of rice blast fungus *M. oryzae*

#### 1.2.4.1 Attachment and germination of conidia

The biology of *M. oryzae* has been intensively studied (for reviews see Wilson and Talbot, 2009). The life cycle of *M. oryzae* is illustrated in Figure 1-2. The rice blast fungus infects rice at all stages of development and affects all above-ground parts of the plant, including leaves, stems, and panicles. It has been demonstrated that *M. oryzae* has the capacity to infect the roots of cereals and move to the aerial parts causing typical rice blast symptoms (Dufresne & Osbourn, 2001; Sesma & Osbourn, 2004) The fungus produces conidia that attach themselves to the leaf by means of spore tip mucilage (STM) that is produced by the apical cell of the conidium, thus initiating infection (Wilson and Talbot, 2009). Production of spore tip mucilage (STM) occurs before the germ tube emerges and it has been shown that this adhesive secretion firmly attaches the conidium to hydrophobic surfaces such as Teflon, and is effective enough to

resist physical removal of conidia. STM is stored in a compact, unhydrated form in the periplasmic space of dormant conidia (Hamer *et al.,* 1988). Attachment can be blocked by addition of concanavalin A, a lectin that binds to α-D-mannose and α-D-glucose (Hamer *et al.,* 1988; Xiao *et al.,* 1994). A hydrophobin encoding gene, *MPG1* has been identified and characterised and is highly expressed during the initial stages of appressorium formation (Talbot *et al.,* 1993; Talbot *et al.,* 1996). The Mpg1 protein has characteristics typical of a class 1 hydrophobin and directs formation of a rodlet layer on conidia, which contributes to their surface hydrophobicity (Talbot *et al.,* 1993; Talbot *et al.,* 1996). Mpg1 appears to interact with hydrophobic surfaces acting as a developmental cue for appressorium formation. Hydrophobins are small amphipathic proteins that act by reducing surface tension thus allowing production of aerial hypha and spores (Kershaw and Talbot, 1998). In addition, they coat the surface of spores to reduce wettability allowing surface interactions. Recently, the roles of Mpg1 and Mph1, a class II hydrophobin, have been further demonstrated (Pham *et al.,* 2016). The hydrophobins are involved in surface perception and attachment of conidia on the leaf surface and further initiate the infection process by cutin hydrolysis. It has been demonstrated that Mpg1 spontaneously assembles into amyloid-like assemblies while Mph1 assembles into a non-fibrillar film, and that the assembly of hydrophobins and their interactions with enzyme cutinase 2 (Cut2) are crucial functions that direct how *M. oryzae* develops at the infection interface. Cut2 is a methylesterase enzyme required for breakdown of cutin that covers the leaf surface and is required for full pathogenicity (Skamnioti & Gurr, 2007). An interplay between Cut2 and the hydrophobins has been reported and it has been shown that Mpg1 rodlets may enhance the activity of cutinase through localisation

of the protein, or activation of the protein through conformational changes (Pham *et al*., 2016).

#### 1.2.4.2 Appressorium formation and morphogenesis

Once attached to the leaf surface, a germ tube emerges, grows across the leaf surface, swells at the tip, and becomes flattened against the leaf surface (Talbot, 2003). This hooking process precedes formation of a dome-shaped infection cell, called an appressorium (Howard and Valent, 1996). The presence of a hard, hydrophobic surface, the presence of cutin monomers and nitrogen starvation, are all environmental cues that lead to formation of the appressorium (Uchiyama & Okuyama, 1990; Jelitto *et al.*, 1994; Talbot *et al*., 1997). During germination of conidia, a single round of mitosis occurs and one daughter nucleus migrates into the swollen germ tube tip, which stops growing and expands isotropically to form an appressorium, while the other nucleus migrates back into the conidium (Veneault-Fourrey *et al.,* 2006). Saunders *et al.* ( 2010) demonstrated that entry of the infection cell into synthesis phase (S-phase) of the cell cycle is critical for initiation of appressorium development and entry into mitosis represents a commitment point for appressorium morphogenesis. Findings by Saunders *et al.* (2010b) indicate that nuclear division and cytokinesis are spatially separated and that migration of the nucleus into the swollen hyphal tip always occurs before differentiation and cytokinesis of the appressorium. A recent study (Osés-Ruiz *et al.,* 2017), has clarified the role of cell cycle control in appressorium development and maturation. The study provides evidence that two distinct S-phase checkpoints operate over two successive cell cycles in *M. oryzae* and are required for appressorium formation, maturation and formation of penetration peg. The first checkpoint operates at the initial stage of appressorium formation and is controlled by a serine threonine protein kinase, Cds1, which is involved in

the DNA damage response (DDR) pathway. During DNA damage, the cell cycle is inhibited by phosphorylation of the B-cyclin-CDK1 complex, which is mediated by Cds1. In presence of a DNA synthesis inhibitor, hydroxyurea (HU), appressorium morphogenesis proceeds normally in Δ*cds1* deletion mutants indicating that the checkpoint involves the DDR pathway. By contrast, the second checkpoint is independent of the DDR pathway and controlled by a novel mechanism that involves turgor pressure generation within the appressorium. The second checkpoint controls NADPH oxidase-activated, septin-dependent F-actin remodelling at the base of the appressorium and thus is important for repolarisation of the infection cell and formation of a penetration peg. The generation of a minimum threshold turgor is necessary for progression into the second S-phase checkpoint because melanin-deficient mutants, which are normally impaired in turgor generation, produce appressoria which remain arrested in the Gap1 (G1) phase of the cell cycle (Osés-Ruiz *et al.,* 2017). Completion of mitosis leads to autophagic collapse of the conidium and its cell contents are then delivered into the appressorium (Veneault-Fourrey *et al.,* 2006).

Development of the appressorium is linked to re-modelling of the actin cytoskeleton, which is mediated by septin GTPases and cell differentiation. These processes are regulated by environmental cues and cell cycle control, and for appressorium to form, mitosis, nuclear migration and autophagy are all required (Veneault-Fourrey *et al.,* 2006; Kershaw & Talbot 2009; Ryder & Talbot, 2015) A genome-wide study by Kershaw & Talbot (2009) demonstrated that autophagy is necessary for both conidial cell death, maturation and differentiation of the appressorium. Systematic targeted deletion of 16 different *ATG* genes

involved in macroautophagy in *M. oryzae* rendered the mutants non-pathogenic or weakly pathogenic, and unable to penetrate the rice leaf surface.

In fungi, mitogen-activated protein kinase (MAPK) cascades and the calcium–calcineurin pathway are important in controlling virulence in fungi and are functionally conserved in a wide range of taxonomic groups (Rispail *et al.,* 2009). MAPK kinase cascades contain three-tiered protein kinases that operate sequentially: a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MEK) and a MAP kinase (MAPK) (Rispail *et al.,* 2009). MAPKs operate with upstream kinases in order to transmit environmental signals from the surface of a cell to the nucleus, thereby leading to expression of genes (Talbot, 2003). In *M. oryzae,* the MAPK Pmk1 is required for appressorium development and pathogenicity, with deletion mutants being defective in appressorium formation and infection. However, *Δpmk1* deletion mutants are able to recognise hydrophobic surfaces and respond to exogenous cyclic AMP/. (cAMP), forming hooked germ tubes (Xu & Hamer, 1996). Moreover, the MAP kinase cascade also consists of *MST11* (MAPKKK) and *MST7* (MAPKK), which act upstream of *PMK1* and are required for appressorium formation (Zhao *et al.,* 2005). Recently, Sakulkoo, (2016) has demonstrated that *PMK1* is a central regulator of several infection-related processes including appressorium development, plant penetration, and tissue colonisation.

The cyclic AMP signalling pathway is involved in regulating plant infection, controlling appressorium maturation and turgor-driven infection. Disruption of a gene encoding the catalytic subunit of cAMP-dependent protein kinase A (Cpka) inhibited appressorium formation and the responsiveness of germinating conidia to exogenous cAMP. The *Δcpka* mutant is non-pathogenic even when inoculated

by wounding rice plants (Xu *et al.,* 1997). Both the Pmk1 MAP kinase pathway and cAMP-dependent protein kinase A pathway positively regulate appressorium morphogenesis (Yan & Talbot, 2016).

Once developed, the appressorium forms a thick melanin layer on the inner side of the cell wall, which allows the cell to develop high turgor pressure from accumulation of solutes, such as glycerol, which are prevented from leaking from the cell due to the low porosity of the appressorium cell wall (Talbot, 2003). Once a threshold pressure has been attained in the appressorium, re-modelling of the actin cytoskeleton occurs, mediated by septin GTPases, leading to organisation of F-actin at the base of the appressorium to form a specialised zone known as the appressorium pore (Dagdas *et al.*, 2012). The appressorium pore is the point at which the fungus secretes, into rice cells, proteins such as enzymes and effectors during the early phases of the host-pathogen interaction (Yan & Talbot, 2016).

The septins organise the F-actin network to form a toroidal network which gives cortical rigidity to the appressorium and also acts as a diffusion barrier to ensure localization of proteins, such as the Rvs167 BAR protein, and the WASP/WAVE complex that are involved in membrane curvature at the tip of the emerging penetration peg (Dagdas *et al.*, 2012). Septin-mediated reorganisation of the F actin network is mediated by NADPH oxidases (Nox). The Nox2–NoxR complex is required to spatially organize the heteroligomeric septin ring, whilst Nox1 is required for elongation of the penetration hypha (Ryder *et al.,* 2013).

Once the appressorium has developed and become melanized, a penetration peg emerges and exerts pressure that ruptures the host surface to facilitate entry of the fungus into leaf epidermal cells (Howard and Valent, 1996). In root

infections, which have been observed in *M. oryzae* under laboratory conditions it has been shown that melanisation is not required for infection to take place (Dufresne & Osbourn, 2001; Sesma & Osbourn, 2004). During root infection by *M. oryzae* and similar to root-infecting pathogens, such as *Gaeumannomyces graminis*, hyphae swell to form a simple infection structure called a hyphopodium (Dufresne & Osbourn, 2001; Sesma & Osbourn, 2004). In addition, microsclerotia which are typical of root-infecting fungi, can be observed on the root surface.

In *M. oryzae,* a second MAPK, designated Mps1 is functionally related to the yeast Slt2 kinase and is essential for the maintenance of cell-wall integrity during specific phases of the life cycle, especially during turgor-driven penetration of plant cells. In the budding yeast *Saccharomyces cerevisiae,* cell wall integrity during membrane stress is maintained by a protein kinase C pathway that activates the mitogen-activated protein kinase (MAPK) Slt2yMpk1. Δ*Mps1* mutants are completely non-pathogenic due to an inability of the appressorium to rupture the cell surface, suggesting that penetration requires re-modelling of the appressorium wall through an *MPS1*-dependent signalling pathway (Xu *et al.,* 1998). Recently, it has been shown that *MPS1* indirectly regulates expression of a transcription factor-encoding gene*, MOGTI1,* required for maintaining cell wall integrity, conidiation and plant infection (Li *et al.,* 2016).

#### 1.2.4.3 Invasive growth by *M. oryzae* in the rice cells

In the first invaded plant cell, the primary hypha and filamentous invasive hypha (IH) differentiate into bulbous IH, which are surrounded by a host-derived extra-invasive hyphal membrane (EIHM). The IH undergo biotrophic growth, filling the first cell, and locating plasmodesmata, where hyphae severely constrict and uses the Pit field to move to the next cell. The fungus secretes several effectors to

suppress host defence mechanisms and these appear to manipulate the structure and function of plasmodesmata to allow fungal proliferation in host rice cells (Kankanala *et al*., 2007). Once inside rice tissue, the fungus initially grows as a biotroph, drawing nutrients from living cells by means of bulbous, branched hyphae that occupy epidermal cells and spread throughout the mesophyll tissue intracellularly. The fungus grows extensively as a biotroph, before switching to necrotrophic growth, killing plant cells and preparing for sporulation from disease lesions (Talbot, 1995).

In order for *M. oryzae* to proliferate in plant cells and complete its lifecycle, the fungus secretes effector proteins that interfere with host immunity to allow the pathogen to invade host cells. This is known as effector-triggered susceptibility and is responsible for suppression of the initial line of plant defence, known as PAMP-triggered immunity, in which host pattern recognition receptors can perceive pathogen-associated molecular patterns released by the invading pathogen. In the case of fungi, these include chitin which in rice is perceived by the chitin elicitor binding protein (CEBiP), which triggers a host defence response (Jones and Dangl, 2006). In *M.* oryzae, the secreted LysM Protein1 (Slp1) effector, competes with CEBiP for binding of chitin oligosaccharides leading to suppression of chitin induced plant defence responses including generation of reactive oxygen species and expression of plant defence genes (Mentlak *et al.,* 2012). Fungal effectors can, however, be recognised by host immune receptors, which are the products of plant disease resistance genes (R-genes). These effectors are then known as Avr proteins which are effectors encoded by avirulence genes (*AVR*) and recognised by the products of corresponding plant R genes, leading to race-specific recognition. In *M. oryzae*, over 40 *AVR* genes, involved in rice blast immunity, have been identified and 11 have been cloned

(Huang *et al*., 2014). With the exception of *ACE1* that encodes an intracellular hybrid PKS-NRPS protein, other cloned *AVR* genes encode secreted proteins which are expressed in invasive hyphae. Avr proteins are involved in suppressing host immunity to allow proliferation of the fungus in the plant. Avr-Pizt, for example, suppresses ubiquitination by inhibiting ubiquitin ligase activity of the rice RING E3 ubiquitin ligase AP1P6 (Park *et al.,* 2012). Avr-Pizt also promotes degradation of APiP6 and APiP10 and suppresses transcriptional activity of APiP5. Avr-proteins may also be associated with plant defence-associated membrane trafficking. For example, Avr-Pii has been shown to bind to two rice Exo70 proteins, OsExo70-F2 and OsExo70-F3, which are presumed to be involved in exocytosis. Simultaneous knockdown of OsExo70-F2 and OsExo70-F3 resulted in loss of the Pii-activated immune response (Fujisaki *et al*., 2015). It has also been established that some R-gene products contain an integrated domain that consists of protein domains associated with effectors. For example in *M. oryzae*, two NB-LRR protein coding genes, *RGA4* and *RGA5*, are involved in recognition of Avr-Co39 and unrelated effector protein, Avr-Pia*,* by physically binding to Rga5, which has an integrated HMA (heavy metal-associated) protein domain. Similarly, the rice blast resistance gene *Pik*, which recognizes the Avr-Pik contains an integrated HMA domain (Maqbool *et al.* 2015) This also indicates that multiple R genes may have multiple specificities (Cesari *et al.,* 2013).

For effector proteins to functionally suppress host immunity they must be secreted into rice cells. There are two pathways by which *M. oryzae* secretes effector proteins into rice cells (i) Cytoplasmic effectors, destined for delivery into rice cells, accumulate in the biotrophic interfacial complex (BIC) before being delivered into rice plant cells. For example, Avr-Pita, Pwl1, Pwl2, Bas2 and Avr-Piz-t all localize to the BIC during invasive hyphal growth. The BIC is a distinct

plant-derived, membrane-rich structure which develops at the tip of the primary IH. (ii) Apoplastic effectors are secreted from hyphal tips into the extracellular space between the fungal cell wall and the EIHM. For example, Bas4 and Slp1 are secreted into the apoplast (Zhang & Xu, 2014). In addition, cytoplasmic and apoplastic effectors use distinct systems for secretion. Apoplastic effectors utilise the Golgi-dependent conventional secretory pathway whereas, cytoplasmic effectors appear to utilise a novel system involving the exocyst that is independent of the Golgi-dependent pathway (Giraldo *et al.,* 2013).

*M. oryzae* also appears to suppress rice immunity by manipulating hormone-regulated plant defence. During host invasion and colonisation, it is known that fungal pathogens can target phyto-hormones directly involved in host defence responses, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). Additionally they may also modulate growth hormones such as indole-3-acetic acid (IAA), abscissic acid (ABA), cytokinin (CK), or gibberellin (GA), which may regulate immune signalling in plants (Patkar & Naqvi, 2017). In rice, CK hormone is involved in controlling key developmental processes like source/sink distribution, cell division or programmed cell-death and its manipulation has been shown to play an important role in conferring full pathogenicity to *M. oryzae.* In *M. oryzae,* CK is produced by the fungus using the Cytokinin Synthesis 1 (*CKS1*) gene and fungal deletion mutants are severely affected in growth and pathogenicity (Patkar & Naqvi, 2017). Recently, Patkar *et al.* (2017) demonstrated that *M. oryzae* produces and secretes an antibiotic biosynthesis monooxygenase (Abm) which is an analogue to a phytohormone and plays an important role in modulating host immunity by blocking JA-mediated signalling.

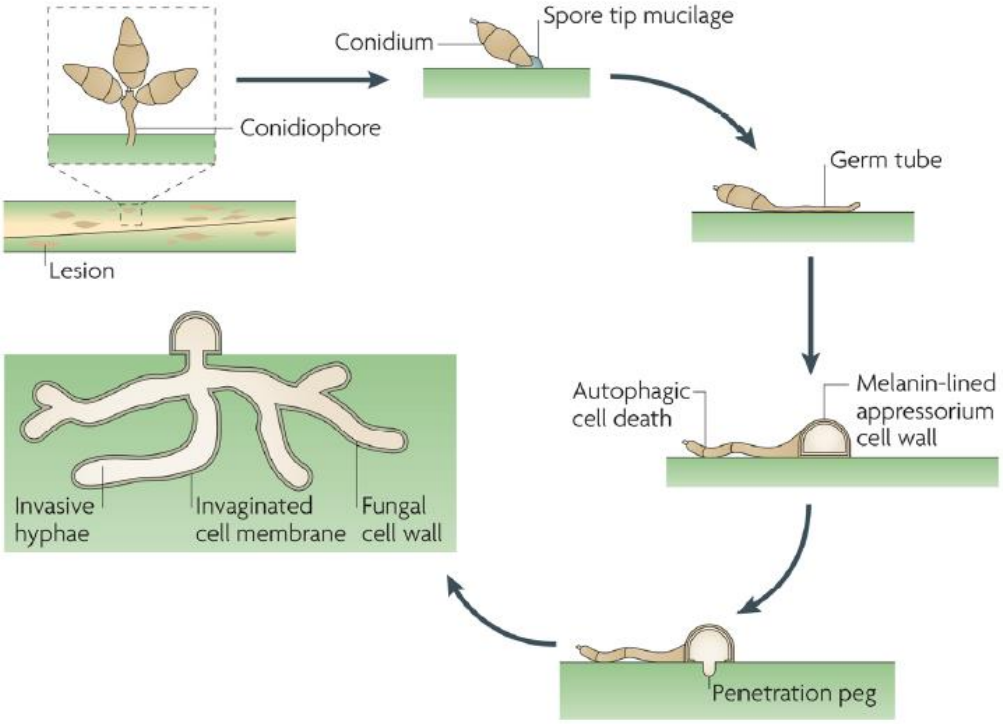


Figure 1-2. Life cycle of the rice blast fungus *M. oryzae*

A three-celled conidium lands on the leaf surface and attaches itself to the hydrophobic cuticle before initiating germination. The conidium germinates and produces a germ tube which hooks at its tip, and then differentiates into an appressorium. The appressorium becomes melanised, generates internal turgor pressure and a penetration peg is formed at the base, which subsequently punctures the cuticle allowing entry into the rice epidermis. The appressorium matures and conidium collapses and dies in a programmed process, involving autophagy. Invasion of rice occurs by means of bulbous invasive hyphae that invaginate the rice plasma membrane and invade epidermal cells. Cell-to-cell movement occurs through Pit fields where plasmodesmatal channels are located. Disease lesions occurs between 72-96h with sporulation occurring under humid conditions. Sympodially-arrayed spores are produced in the aerial conidiophores and spread to new hosts by wind or water splashes (Talbot & Wilson, 2009).

### 1.2.5 Rice blast disease management

The rice blast fungus occurs in all rice growing areas of the world, has a wide host range and is genetically variable. Therefore, many control practices useful in reducing other plant diseases are of limited use to control rice blast (Pooja & Katoch, 2014). Rice blast management strategies can be broadly classified into the following:

#### 1.2.5.1 Cultural control

Manipulation of cultural practices is one of the most widely used control measures for management of rice blast disease. Although the effectiveness of cultural practices is limited when used alone, they play an important role in integrated rice blast management (Mew, 2016).

**Nutrient management**

The rate, frequency and timing of nitrogen application has been shown to have an influence on rice blast severity. Studies have shown that excess application of nitrogen favours development of rice blast (Kürschner *et al.,* 1992; Mukherjee *et al.,* 2005; Prabhu *et al.,* 1996; Pooja & Katoch, 2014). It has been suggested that high disease incidence observed under high nitrogen regimes may be due to increased susceptibility and increased canopy density that creates a favourable micro-climate for rice blast disease development (Pooja & Katoch, 2014). In upland rice, Prabhu *et al.* (1996) reported higher rice disease severity at 60 kg/hectare (ha) of nitrogen application compared to 10 Kg/ha. Similarly, Kürschner *et al.* (1992) reported suppression of leaf blast disease when nitrogen (90 Kg per hectare) was applied in two equal late splits at 30 and 60 days, respectively after seeding, compared with early application. The study showed that rice blast was consistently suppressed when nitrogen was not applied at planting. Further, it has been shown that rate, frequency and timing of nitrogen

application has a greater influence on leaf blast than panicle blast and their effects are more pronounced on susceptible than tolerant rice varieties. Taken together, these studies suggest that optimum amounts of nitrogen for optimum yield and rice blast management must be impirically determined since soil ferility varies in space and time.

Silicon is the second most abundant mineral in the soil, constituting about 28% of the earth's surface layers and although it is not considered as an essential nutrient in higher plants, its concentration in plant tissues is equivalent to that of macronutrients. Silicon plays an important role in improving plant growth and yields by increasing resistance against environmental stresses and penetration of pathogens. Silicon has also been reported to increase leaf erectness resulting in increased photosynthesis, improve water usage, decrease toxicity of heavy metals and reduces cuticular transpiration (Rodrigues & Datnoff 2005; Rebitanim *et al*., 2015; Ashtiani *et al.,* 2012b). Plants accumulate 1-10% silicon depending on species, with rice reported to accumulate as much as 10% of its dry weight. The rate of silicon accumulation in rice is higher than other essential macroelements, for instance, such as nitrogen, phosphorus and potassium. In paddy farming systems, silicon is supplied by addition of silicon-containing fertilisers. Plants absorb silicon in the form of mono silisic acid $Si(OH)_4$ which accumulates in the cell walls as silica gel. $SiO_2$-$nH_2O$ also referred to as Opals or Phytoliths accumulates in the epidermal cells of rice leaves (Rodrigues & Datnoff, 2005; Rebitanim *et al*., 2015; Ashtiani *et al.*, 2012b).

Several authors have shown that application of silicon-containing ferilisers reduces the severity of rice blast disease (Rebitanim *et al.,* 2015*;* Hayasaka *et al.,* 2005*;* Ashtiani *et al.*, 2012; Datnoff *et al.,* 1991; Kürschner *et al.,* 1992). It has also been reported that rice blast severity is directly related to silicon deficiency

in soil (Ashtiani *et al.,* 2012a). Ashtiani *et al.* (2012b), applied silicon to the soil prior to planting using silica gel (0, 60, 120, 180 g/5 kg of soil) and liquid sodium silicate (0,1,2,3 ml $L^{-1}$). Results indicated that there was a significant reduction in blast disease severity in plants treated with silicon, with the highest reduction (75%) observed in plants treated with 120 g/5 kg of soil. Calcium silicate slag broadcast at rates of 5, 10 and 15 mg/Ha significantly reduced rice blast, for example (Datnoff *et al.,* 1991). Similarly, Hayasaka *et al.* (2005), indicated that a threshold of 5% $SiO_2$ content is required in the leaves of rice seedlings for effective reduction of rice blast disease. Two main mechanisms involved in silicon-mediated rice blast resistance have been suggested. Firstly, that the mechanism of resistance is associated with accumulation and polymerisation of silicic acid in the leaf epidermal cells, resulting in increased density of silicied buriform cells present in the epidermis of rice. This forms a physical barrier blocking penetration of *M. oryzae* into rice cells (Ashtiani *et al.,* 2012; Rodrigues & Datnoff, 2005; Rebitanim *et al.,* 2015). Cytological studies by Kim *et al.* (2002) showed that silicon was deposited in epidermal cells walls, middle lamellae and intercellular spaces within sub-epidermal tissues and silicified epidermal cells were closely associated with significantly reduced rice blast severity.

Secondly, a biochemical role for silicon in enhancing resistance to rice blast has been reported. Rodrigues *et al.* (2003) reported accumulation of diterpenoid phtytoalexins in silicon-treated rice plants which was associated with altered fungal hypha and restricted growth (Rodrigues *et al.,* 2004; Rodrigues *et al.,* 2003). According to Rodrigues *et al.* (2003), cytological studies revealed accumulation of an amorphous material, that stained densely with toluidine blue and reacted positively to osmium tetroxide, was a typical feature of cell reactions to *M. oryzae* in silicon-treated plants. The authors observed that deformed fungal

hypha was surrounded or trapped in amorphous material in silicon-treated plants, suggesting that phenolic-like compounds or phytoalexins played a primary role in rice defense against *M. oryzae.* Taken together, these studies indicate that appropriate management of nitrogen and silicon in soil has the potential to reduce rice blast disease in the field and are therefore important components in integrated rice blast management.

**Water Management**

Availability of water also affects susceptibility of the host plant to *M. oryzae.* Rice grown under upland conditions is more susceptible than rice grown in flooded soils, for example. Rainfed rice grown under drought conditions is susceptible to rice blast (Bonman, 1992; Pooja & Katoch, 2014). The transcriptional basis of drought-induced susceptibility to *M. oryzae* has been studied. Bidzinski *et al.* (2016) set-up an experimental system, displaying mild and intermittent drought stress before inoculation with *M. oryzae,* in order to identify transcriptional and cellular mechanisms underlying drought-induced susceptibility to rice blast. The study indicated that several aspects of basal immunity, including the oxidative burst and transcription of pathogenesis related genes were supressed in drought-stressed plants. The authors used the RiceDB database to retrieve rice R-genes differentially expressed in drought experiments and found that 26 genes were repressed with 11 being induced in expression. Interestingly, RNAseq analysis of global fungal transcription under drought conditions revealed that expression of many fungal genes encoding biotrophic effectors was reduced while expression of genes coding for cell wall degrading enzymes was increased. The authors proposed a model in which unknown plant signals trigger a change in the virulence program of a pathogen to adapt to a plant host affected by drought (Bidzinski *et al.*, 2016). This strategy favours effector-immunity breakdown by

reducing expression of effector genes and/or down-regulating R gene expression.

#### 1.2.5.2 Biological control

In order to maintain optimum rice yields, crop farming has largely relied on application of pesticides to control pests. However, due to health and environmental concerns associated with pesticides, several efforts have been put in place to develop other safe and environmental-friendly disease control strategies (Law *et al.*, 2017). Biological control is one strategy for the sustainable management of pests. Research in biological control of rice diseases began in the 1980s with efforts focused on identification, evaluation and formulation of biopesticides (Manidipa *et al.,* 2013). The potential of utilising biological control in management of rice blast has been evaluated using different groups of biocontrol organisms. Three main biocontrol agents (BCA) that have been evaluated for management of rice blast include the following:

**Pseudomonads as potential biocontrol agents of rice blast**

Plant growth promoting rhizobacteria (PGPR) are root-associated bacteria that colonise plant roots and consequently influence plant health and soil fertility. Among the PGPRs, fluorescent *Pseudomonas* are the largest and most promising plant disease biocontrol agents due to their rapid growth, simple nutritional requirements, ability to utilise diverse organic substrates, and motility (Dorjey *et al.*, 2017). Fluorescent pseudomonads are gram negative, aerobic motile rods with polar flagella, have the ability to produce water soluble yellow green pigment, and are fluorescent under low wave length ultra violet radiation (Manidipa *et al.,* 2013; Dorjey *et al.,* 2017). Fluorescent pseudomonads have been commercially utilised as biocontrol agents against field and postharvest diseases including postharvest fungal diseases. In the USA, *P. fluorescens,*

strain A506 under the trade name BlightBan A506 has been utilised to manage fire blight disease in pears and apples (Stockwell & Stack, 2007). Combining BlightBan A506 with the antibiotic streptomycin, improved control of fire blight, even in areas with streptomycin-resistant populations of the pathogen. According to Pesticide Control Products Board of Kenya (2018) *P. fluorescens* is registered under the trade name Biocure B 1.75WP and is utilised for management of *Botrytis*, septoria leafspot (caused by *Zymoseptoria tritici*) and *Sclerotonia* (Pesticide Control Products Board of Kenya, 2018). *P. fluorescens* also has the potential to control rice blast (Krishnamurthy & Gnanamanickam, 1998; De Vleesschauwer *et al.,* 2008). Studies by Krishnamurthy & Gnanamanickam (1998) show that when *P. fluorescens* strain Pf7-14 was applied as a seed treatment followed by three foliar applications, the strain provided 68.5% suppression of rice blast in the seedbed and 59.6% in field experiments. Furthermore, persistence and migration of *P. fluorescens* strain Pf7-14 on rice plants was monitored with the aid of *lacZY* genes expressed in the bacterium. Results showed that migration of Pf7-14 from seed to leaves occurred only until the seedlings were 16 days old, whilst when applied by spray Pf7-14 was detected in the leaves for the next 40 days. However, the bacteria persisted in roots of rice for 110 days, the length of the cropping period. Due to limited migration and persistence of Pf7-14 on leaves, the authors suggested the need for multiple foliar application to sustain adequate bacterial population for the effective suppression of rice blast. Similarly, results by Kumar *et al*. (2017) show that application of *P. fluorescens* reduced rice blast severity by 21.9% under field conditions.

### *Streptomyces* as a potential biocontrol agent of rice blast

The genus *Streptomyces* consist of about 700 species. *Streptomyces* species are gram-positive, aerobic, non-motile, catalase positive, and non-acid-fast bacteria with a filamentous form (Law *et al.,* 2017). Greenhouse studies undertaken by Li *et al.* (2011) and Zarandi *et al.* (2009) indicated that *Streptomyces* has the potential to suppress rice blast. Rice plants treated with *S*. *sindeneusis* isolate 263 showed significantly lower lesion development with only 0.5% of the leaf infected compared to 8% in the untreated controls (Zarandi *et al.,* 2009). When culture filtrate of *S. globisporus* strain JK-1 was applied to rice seedlings in the greenhouse and challenged with *M. oryzae* 2h later, an 88.3% disease reduction was observed when compared with untreated plants. Furthermore, microscopy observations revealed that conidial germination and appressorium formation were greatly inhibited by the culture filtrate. Findings from these studies indicate that *S*. *sindeneusis* isolate 263 and *S. globisporus* JK-1 had the same level of efficacy as tricyclazole and blasticidin-S fungicides in suppressing rice blast (Zarandi *et al.,* 2009).

### *Trichoderma* spp as potential biocontrol agents of rice blast

The genus *Trichoderma* belongs to the phylum Ascomycotina, class Sordariomycetes, order Hypocreales, family Hypocreaceae (Li *et al.,*2011). *Trichoderma* spp. are the most frequently isolated fungi from soil and plant roots and are opportunistic plant symbionts that function as parasites and antagonists of many plant pathogens (Vinale *et al.,* 2008). *Trichoderma* spp. are among the most studied and commercially marketed fungal biocontrol agents with over 50 formulations registered worldwide, accounting for about 60% of fungal biocontrol agents marketed (Rajesh *et al.,* 2016) . *Trichoderma* spp. have been used in many crops and ornamentals to control soil-borne and foliar pathogens, including

*Fusarium* spp.*, Pythium* spp., *Rhizoctonia solani*, *Sclerotonia* spp.*, Phytophthora* spp., *Botrytis cinera, Leptosphaeria maculans* among others (Rajesh *et al.*, 2016; Błaszczyk *et al.,* 2014). In Kenya, various *Trichoderma* based products are registered for management of pests, including root knot nematodes, soil-borne fungal diseases caused by *Pythium* spp.*, Fusarium* spp.*,* and *Rhizoctonia* spp. (Pesticide Control Products Board of Kenya, 2018). It has been shown that rice seed treated with *Trichoderma* improved seed germination, reduced the time to flowering and reduced rice blast severity (Singh *et al.,* 2012). Several mechanisms are employed by biocontrol agents to suppress plant pathogens including:

**Mycoparasitism and lytic enzymes**

Mycoparasitism is the main mechanism employed by *Trichoderma* spp. to enhance its survival and includes recognition of the host, attack and subsequent penetration and killing. During mycoparasitim, *Trichoderma* spp. secretes cell wall degrading enzymes (CWDE) that digest the cell walls of the host fungi leading to release of oligomers from the cell wall. The degraded cell wall components act as a cues for detection of the host fungus and activation of cascade of genes involved in secretion of CWDE (Vinale e*t al.,* 2008).

**Antibiosis and secondary metabolism**

Fluorescent pseudomonads, *Trichoderma* spp. and *Streptomyces* spp. produce antibiotics and other secondary metabolic products that have an inhibitory effect on phytopathogens. Secondary products includes a group of heterogeneous chemically-distinct natural compounds that are related to survival functions of the producing organisms (Vinale e*t al.,* 2008). Antibiotic production is correlated with biocontrol activity and it has been shown that application of purified antibiotics exert a similar biocontrol activity to corresponding living microbes (Ghisalberti &

Sivasithamparam, 1991). The production of secondary metabolites in *Trichoderma* spp. is strain dependent and includes a variety of antifungal compounds broadly categorised into three groups (i) volatile antibiotics, for example, 6-pentyl-a-pyrone (6PP) and most of the isocyanide derivatives that have a relatively long distance range of influence on soil microbiota (ii) water-soluble compounds, i.e. heptelidic acid or koningic acid (iii) peptaibols, which are linear oligopeptides of 12–22 amino acids rich in a-aminoisobutyric acid, N-acetylated at the N-terminus and contains an amino alcohol (Pheol or Trpol) at the C-terminus (Vinale e*t al.,* 2008).

Fluorescent pseudomonads also produce a range of antibiotics that are lethal to plant pathogens. A phenolic compound with antibiotic properties, 2,4-diacetylphloroglucinol (DAPG), is produced by most pseudomonads. Several strains of fluorescent pseudomonads also produce phenazines, which comprise of a large family of heterocyclic nitrogen-containing, coloured pigments. In addition, both fluorescent and non-fluorescent pseudomonads produce Pyrrolnitrin, which is a broad spectrum anti-fungal metabolite that is known to persist in soil for at least 30 days (Manidipa *et al.,* 2013; Dorjey *et al.,* 2017). Hydrocyanic acid (HCN) plays a major role in suppression of phytopathogens by fluorescent pseudomonads. HCN inhibits activity of cytochrome oxidase and electron transport thereby affecting energy supply to the cell. Furthermore HCN has been shown to degrade fungal cell walls (Manidipa *et al.,* 2013). *Streptomyces* spp. are prolific producers of antibiotics that have been utilised in medicine and pest control products. For example, well known fungicides for control of rice blast in Japan, such as Blasticidin-S and Kasugamycin, were isolated from *S. griseochromogenes* and *S. kasugaensis,* respectively. Other

antibiotics of agricultural importance produced by *Streptomyces* spp. include Rapamycin and Pyrroles (Law *et al.,* 2017).

**Competition with pathogens and soil microbial community**

Competition for nutrients and space or infection sites, may be used by biocontrol agents to control pathogens. For example, *T. harzianum* is able to control *Botrytis cinerea* on grape fruits by colonising infection sites (Sivan & Chet, 1989). *Trichoderma* has a strong ability to mobilise and take up nutrients therefore making it more competitive than other soil microbes. Control of *Fusarium oxysporum* f.sp. *melonis* by *T. harzianum* has been attributed to competition for nutrients (Vinale e*t al.,* 2008). Siderophores are low molecular weight ferric compounds that have high affinity for iron and are secreted extracellularly under iron-limiting conditions in order to chelate iron and make it available to cells. Formation of siderophores by fluorescent pseudomonads and to a lesser extent by *Trichoderma* spp. is a mechanism employed to chelate iron from the soil thereby making it less available to other soil microbes. Transport of iron into the cell is mediated by membrane receptors that specifically recognise the ferric-siderophore complex (Manidipa *et al.,* 2013; Dorjey *et al.,* 2017).

### 1.2.5.3 Induction of plant defence responses

Elicitation of induced plant resistance depends on the host/bacterium combination. For example, *P. fluorescens* WCS374r elicits induced resistance in *Arabidopsis* and not in radish or carnation (Pieterse *et al.,* 2001). Induced systemic resistance (ISR) in *Arabidopsis* is elicited by bacterial lipopolysaccharides (LPS) and although ISR resembles pathogen acquired systemic resistance (SAR), it triggers a different pathway that does not require salicylic acid (SA). ISR induced by *P. fluorescens* WCS374r on *Arabidopsis* is independent of SA accumulation and pathogenesis-related gene activation but

requires response of jasmonic acid and ethylene. Simultaneous induction of ISR and SAR results in elevated levels of protection (Pieterse *et al.,* 2001). Induction of plant resistance by colonisation with *Trichoderma* is similar to that induced by rhizobacteria and is elicited by various metabolites including (i) proteins with enzymatic activity e.g., xylanases (ii) low molecular weight compounds released during degradation of cell wall components by *Trichoderma* spp. (iii) avirulence-like gene products released by *Trichoderma* spp. (Vinale e*t al.,* 2008).

#### 1.2.5.4 Plant growth promoting activity

Many BCA promote plant growth and development leading to improved plant disease resistance. For example, in greenhouse and field trials *T. harzianum* and *T. atroviride* enhanced growth of lettuce, tomato and pepper with crop productivity increasing by up to 300%, compared to untreated plants (Vinale e*t al.,* 2008). Plant growth-promoting rhizobacteria promote plant growth by different mechanisms including (i) phosphate solubilisation (ii) nitrogen fixation (iii) production of indole-3- acetic acid (iv) siderophore formation (v) degradation of environmental pollutants (Gouda *et al.,* 2018).

Taken together, these studies show that biological control has a potential to protect rice from rice blast disease. Although biopesticide use is increasing by almost 10% globally, biopesticides comprise only 5% of total crop protection market globally, with a value of $3 billion worldwide. The global market has to grow further for biopesticides to play a significant role in crop protection and reduce over-reliance on synthetic chemicals (Damalas & Koutroubas, 2018). Various factors contribute to low adoption of biopesticides for pest management including (i) lack of awareness of benefits of biopesticides by farmers (ii) lack of farmer confidence because of inconsistent field performance. Rapid decline in inoculated populations and failure to maintain sufficient activity over a prolonged

period of time is a major constraint application of biopesticides. The rapid decline in populations has been attributed to adverse abiotic soil factors including textural type, pH, temperature and moisture (iii) efficient introduction of biopesticides into the soil during the growing period (iv) high cost of production and small-scale agribusinesses for biopesticides (v) issues with the regulatory framework for biopesticides. Biopesticides contain live cells and are therefore treated as pathogens by government agencies, making the registration process long and expensive. Furthermore, import and export of biopesticides is under strict regulation (Mishra *et al.,* 2015). According to the authors, future effects must focus on building farmers' confidence by developing stress-tolerant formulations, improving production technology for biopesticides, improving quality control measures and extensive research undertaken to develop appropriate formulations that are stable under field conditions.

#### 1.2.5.5 Chemical rice blast control

A number of fungicides have been used to manage rice blast (Table 1-1). Chemical control of rice blast began in Japan with the use of antibiotics, such as kasugamycin and blasticidin S (Mew *et al.,* 2016). However, resistance to these compounds developed rapidly, leading to introduction of ferimzone in 1992. Japan has one of the most intensive rice production systems, is a major consumer of rice fungicides, and accounts for 50% of the global rice fungicide market with South Korea, Indonesia, and China having smaller but significant markets. In contrast, fungicide use has been low in Africa, India, Latin America, South and South East Asia (Mew *et al.,* 2016). Most of the fungicides used to control rice blast are systemic, meaning that once applied on the leaves they spread to other plant parts and have a residual effect. This characteristic of fungicides is important because it ensures that a minimal number of applications

is used in a growing season. One of the fungicides widely used to control rice blast, Probenazole, functions by eliciting a rapid immune response including enhanced production of antifungal compounds, activation of defence-related enzymes and increased lignification of rice cell walls (Kato, 2001). In Kenya, the following products are registered for rice blast management: Chariot 500 SC (Carbendazim), Megaprode Lock 52.5 (Carbendazim and Prodione) and Nativo SC (Trifloxystrobin and Tebuconazole) (Pesticide Control Products Board of Kenya, 2018).

Although some broad spectrum fungicides are used to control rice blast, several rice blast-specific fungicides are available and their mode of action is based on inhibiting melanin synthesis (Mew *et al.,* 2016), which is critical in the rice blast infection as explained in section 1.2.4.2. These groups of fungicides are important in management of rice blast disease since they are relatively more efficacious and have limited effects on non-target organisms.

**Melanin inhibitors fungicides for rice blast control**

Melanin biosynthesis starts from pentaketide synthesis and cyclization to form 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), by the action of enzyme polyketide synthase (PKS). This is followed by reduction of 1,3,6,8-THN to scytalone and dehydration of scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-THN). Reduction of 1,3,8-THN to vermelone and dehydration of vermelone to 1,8-dihydroxynaphthalene (1,8-DHN) then occurs. 1,8-DHN is then polymerized and oxidized to yield melanin (Hamada *et al.,* 2014). Melanin inhibitors are specifically used to control rice blast by preventing accumulation of 1,8-DHN in the appressorium requiring chemicals such as tricyclazole (TCZ), Pyroquilon (PRQ) and carpropamid (CAR), or Dichlomycet (DCM). These chemicals interfere with enzymatic reductase and dehydration processes which are key in DHN melanin

biosynthesis. Reductase inhibitors, for example TCZ and PRQ, have a planar structure of fused bicyclic or tricyclic rings and competitively interfere with the binding of planar bicyclic substrates. TCZ and PRQ were registered in Japan in 1981 and 1985, respectively, as granule formulations for use in rice nurseries and paddy fields. Dehydration inhibitors include CAR, DCM, Fenoxanil and cyclobutane carboxamide.

Table 1-1. List of fungicides used in management of rice blast

| **Active ingredient** | **Nature** | **Target site** | **Other biological activity** | **Year[2]** | **Risk of Fungal resistance[1]** |
|---|---|---|---|---|---|
| Probenazole | Systemic | Activates plant defence responses | Rice bacterial blight control, Rice brown spot | 1975 | Not known |
| Tricyclazole | Systemic | Reductase inhibitor in melanin biosynthesis | None | 1976 | Not known |
| Pyroquilon | Systemic | Reductase inhibitor in melanin biosynthesis | None | - | Not known |
| Diclocymet | Systemic | Reductase inhibitor in melanin biosynthesis | Rice water weevil; Rice-stem borer. | 1998 | Not known |
| Fthalide | Systemic | Reductase inhibitor in melanin biosynthesis | None | 1971 | Not known |
| Carpropamid | Root-systemic | Dehydratase inhibitor in melanin biosynthesis | None | 1996 | Medium |
| Fenoxanil | Systemic | Dehydratase inhibitor in melanin biosynthesis | none | 2000 | Medium |
| Tolprocarb | Systemic | Polyketide synthase enzyme (PKS) inhibitor | None | 2012 | Not known |
| Azoxystrobin | Systemic | Quinone outside Inhibitors (QOI) complex 3 inhibitor | Broad spectrum fungal control | 1992 | High. Cross resistance known between all members of the QoI group |

| | | | | | |
|---|---|---|---|---|---|
| Metominostrobin | Systemic | Quinone outside Inhibitors (QOI) complex 3 inhibitor | None | 1998 | High. Cross resistance known between all members of the QoI group |
| Kresoxim methyl | Contact and systemic (local) | Quinone outside Inhibitors (QOI) complex 3 inhibitor | Broad spectrum activity | 1996 | High. Cross resistance shown between all members of the QoI group |
| Propiconazole | Systemic | Demethylation Inhibitors | Wheat rust, leaf and spot blotch | 1980 | Medium. Cross resistance reported in some members. |
| Hexaconazole | Systemic | Demethylation Inhibitors | Seed-borne and soil-borne diseases specially *Ascomycetes* and *Basidiomycetes* spp | 1986 | Medium. Cross resistance reported in some members. |
| Tebucanozole | Systemic | Demethylation Inhibitors | Broad spectrum | 1988 | Medium. Cross resistance known in some members |
| Edifenphos | Systemic | Inhibits biosynthesis of phosphatidylcholine | Helminthosporium, Fusarium control | 1966 | Low to medium. |
| Iprobenfos (IBP) | Systemic | Inhibits biosynthesis of phosphatidylcholine | Rice sheath blight<br>Plant lodging inhibitor | 1967 | Low to medium. |
| Isoprothiolane | | Inhibits biosynthesis of phosphatidylcholine | Rice stem rot, Fusarium leaf spot, Planthoppers | 1975 | Low to medium. |
| Zineb | Contact | Multi-site activity | Broad spectrum | 1950 | Low. |

| | | | | | |
|---|---|---|---|---|---|
| Carbendazim with Mancozeb | Systemic and contact | Carbendazim inhibits spindle formation during mitosis. Mancozeb has multi-site activity | Broad spectrum activity | 1973 and 1961[3] | High risk reported when carbendazim utilised alone. Mancozeb has low risk of resistance. Mixture reduces risk of resistance development |
| Benomyl | Systemic | Binds to microtubules interfering with cell functions e.g. meiosis and intracellular transportation | Broad spectrum | 1968 | High risk |
| Kasugamycin | Systemic | | Broad spectrum activity on pathogens | 1965 | Medium. |
| Blasticidin S | Systemic | | None | | Low to medium. |
| Ferimzone | Systemic | | Helminthosporium, Cercospora control | 1992 | Not known |

Sources: Srivastava *et al.,* 2017; Hu *et al.,* 2014; Hamada *et al.,* 2014; Lewis *et al.,* 2016; Mew *et al.,* 2016; FRAC 2017

[1] Fungicide Resistance Action Committee (FRAC) assessment for intrinsic risk for resistance evolution to a given fungicide group against fungal pathogen.
NA-information not available.
[2] Year of introduction.
[3] Mancozeb introduced in 1962; Carbendazim in 1973.

Due to heavy use of fungicides, concerns have been raised over the effects on both the environment and human. Moreover, development of resistant strains leading to a withdrawal of some fungicides from the market is a reality (Mew *et al.,* 2016). The Fungicide Resistance Action Group (FRAC, 2014), has categorised *M. oryzae* as a pathogen with a high risk of developing fungicide resistance. This category includes plant pathogens that have evolved resistance to fungicides in a time span sufficiently short to be a serious threat to the commercial success of more than one fungicide class. Isolates resistant to isoprothiolane have been reported (Hu *et al.,* 2014). Resistance to Carpropamid has also been reported, and is thought to be due to point mutation resulting in the Valine75Methionine change in scytalone dehydratase, the primary target of the fungicide (Yamada *et al.,* 2004).

Several practices can be employed to minimise chances of development of resistance to fungicides. These include: (i) avoiding exclusive use of a particular product by mixing or rotating fungicides with different modes of action. In some cases, formulated (pre-packed) mixtures of fungicides are available from manufacturers (ii) restricting number of fungicide applications per season (iii) maintaining manufacturers recommended rates (iv) avoiding unecessary use of systemic fungicides by applying the fungicides prophylactically. Applying systemic fungicides to already sporulating lesions increases chances of selection taking place (v) ensuring integrated disease management strategies are practiced (Brent & Hollomon, 2007).

#### 1.2.5.6 Deployment of resistance genes for rice blast management

The introduction of rice blast genes conferring durable rice blast resistance is one of the most economical and convenient methods of managing rice blast. It has been shown that useful lifespan of resistance to rice blast depends on variability

of the pathogen and the type of resistance genes incorporated (Mew *et al.,* 2016). Over 70 genes and 347 quantative traits loci (QTL) involved in rice blast resistance have been detected (Koide *et al.*, 2009) (Table 1-2). Two major categories of disease resistance in rice have been used to confer resistance to diseases. Qualitative resistance, also referred to as vertical or complete resistance, is mediated by major resistance genes (R) that interact with avirulence genes in a gene-for gene manner as explained in section 1.3. Qualitative resistance is pathogen race specific and its durability is limited due to strong selection pressure imposed by the R genes. On the contrary, quantative resistance, also refered to as horizontal or partial resistance, is mediated by QTLs and confers durable non-race specific resistance. In partial resistance, there is a compatible reaction between the pathogen and the host. However, the level of disease development is slower compared to plants with no partial resistance due to presence of minor genes that maintain disease development to acceptable levels. These minor genes are difficult to identify and characterise due to the epistatic effects of major genes. True resistance is governed by a qualitative gene or major gene while field resistance is governed by quantitative genes, also called minor genes. In many cases genes confering qualitative and quantitative resistance are co-located on linkage maps and these regions are typically rich in genes confering resistance to multiple pathogens (Koide *et al.*, 2009; Mew *et al.,* 2016).

Most of genes involved in rice blast resistance are co-localised in chromosomes 6, 11 and 12. Furthermore, various types of markers linked to these genes have been identified making it possible to utilise them in marker assisted breeding (Koide *et al.*, 2009) as shown in Table 1-2.

Table 1-2. Rice blast resistance genes and tightly linked markers that can be utilised in breeding for blast resistance

| Chr | Gene | Type of linked marker | Name | Map Position | Donor rice | Resistance type |
|---|---|---|---|---|---|---|
| 1 | *Pit* | SNP | | 16.1 | Tjhaja | Complete |
| | *Pi27(t)* | SSR | Rm151. RN 259 | 28.4-38.8 | Q14 | Complete |
| | *Pitp(t)* | SSR | Rm246 | 114.1 | Tetep | Partial |
| | *Pi35(t)* | SSR | RM1216, RM1003 | 132.0–136.6 | Hokkai 188 | Partial |
| | *Pi37* | SSR | RM302, RM212, FPSM1, FPSM2, FPSM4 | 136.1 | St. No.1 | Complete |
| | | STS | S15628, FSTS1, FSTS2, FSTS3, FSTS4 | | | |
| | *Pi64* | SSR | RM11715, RM11787 | - | Yangmaogu | Complete |
| | *Pish* | RLFP | - | 148.7–154.8 | Shin2 | Complete |
| 2 | *Pid1*(t) | SSR | RM262 | 87.5–89.9 | Digu | Complete |
| | *Pig1*(t) | SSR | RM166, RM208 | 142.0–154.1 | Guangchangzhan | Complete |
| | *Pitq*5 | RLFP | RG520, RZ446B, RZ446A, RG654, RG256 | 150.5–157.9 | Teqing | Complete |
| | *Piy1*(t) | SSR | RM3248, RM20 | 153.2–154.1 | Yanxian 1 | - |
| | Piy2(t) | SSR | RM3248, RM20 | 153.2–154.1 | Yanxian 1 | - |
| | *Pib* | SNP | b213, b28, b2, b3989, Pibdom | 154.1 | Tohoku, IL9, Koshiihikari | Complete |
| | *Pi14*(t) | Isozyme | Amp-1 | 32.6 | Maowang | Complete |
| | *Pi16*(t) | Isozyme | Amp-1 | 34.3 | AUS373, Maowamg | Complete |
| | *Pi-da*(t) | SSR | RM5529, RM211 | 12.6 | Dacca 6 | - |
| 3 | *Pi66*(t) | SSR | RM487, RM16, RM55, RM168 | | AS201 | Partial |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4 | *Pi21* | STS | P702D03-79 | 58.6 | Owarihatamochi | Partial |
| | *Pikur1* | Isozyme | | 86.0 | Kuroka | - |
| | *Pi39*(t) | SSR | RM3843, RM5473 | 107.4–108.2 | Chubu III | - |
| | *Pi*(t) | - | - | 12.2 | P167(1) | - |
| | *Pi5*(t) | RLFP | RG788, RG498 | 12.0 | RIL29 (Morobere kan) | Complete |
| 5 | *Pi26*(t) | RFLP | RG313 | 22.5–24.7 | Azucena | - |
| | *Pi23*(t) | | - | 59.3–99.5 | Sweon 3655 | - |
| | *Pi10* | inDel | OPF62700 | 88.5–102.8 | Tongil | Complete |
| 6 | *Pi22*(t) | RFLP | - | 38.7–41.9 | Sweon 3655 | - |
| | *Pi26* | RFLP | K17, K2123 | 22.5–63.2 | Gumei 2 | Complete |
| | *Pi27*(t) | RFLP | Est-2 | 51.9 | IR64 | - |
| | *Pi40*(t) | SSR | RM3330, RM527 | 54.1–61.6 | IR65482-4-136-2-2 | - |
| | | CAPS | S2539 | | | |
| | *Piz-5* | STS | BS2-Pi9, NBS4-Pi9 | 58.7 | Tadukan | Complete |
| | *Piz* | inDel | z4794 | 58.7 | Zenith | Complete |
| | | SNP | z60510, z5765, z56592, z565962 | | | |
| | *Piz-t* | inDel | z4794 | 58.7 | Toride | Complete |
| | *Pi9* | - | - | 58.7 | 75-1-127 (101141) | Complete |
| | *Pi25* | RFLP | A7-RG456 | 63.2–64.6 | Gumei 2 | - |
| | *Pid2* | CAPS | CAPS1, CAPS8 | 65.8 | Digu | Complete |
| | *Pigm(t)* | CAPS | C26348 | 65.8 | Gumei 4 | - |
| | *Pitq1* | RLFP | RZ682, C236, RG653, RZ508 | 103.0–124.4 | Teqing | Complete |
| | *Pi8* | Isozyme | Amp-3, pgi-2, Amp-3 | 98.0 | Kasalath | Complete |
| | *Pi13*(t) | RLFP | Amp-3 | 74.6–78.2 | Mawong | Complete |

| | | | | | | |
|---|---|---|---|---|---|---|
| | *Pi13* | RLFP | R2123, R538 | 68.1 | Kasalath | - |
| | *Pi2*(t) | RLFP | RG64 | 2.8 | Cultivar 5173 | Complete |
| | *Pi2-2* | SSR | AP5659-3, RM19817 | 58.7 | Jefferson | |
| | *Pi50*(t) | inDel | GDAP51, GDAP16 | 46.8 | | Complete |
| | *Pi40*(t) | SSR | RM3330, RM527 | 54.1–61.6 | CO39, IR50 | - |
| | | CAPS | S2539 | | | - |
| | *Pi59*(t) | SSR | RM19835 | | Haoru × US-2 | - |
| 7 | *Pi17(*t) | - | - | 94.0–104.0 | Kasalath | Complete |
| 8 | *Pi36* | SSR | RM5647 | 21.6–25.2 | Q61 | - |
| | | CAPS | CRG2, CRG3, CRG4 | | | |
| | *Pi33* | SSR | RM72, RM44 | 45.4 | IR64, Bala | Complete |
| | *Pizh/Pi11*(t) | RFLP | RZ617 | 53.2–84.8 | Zhaiyeqing | Complete |
| | *Pi29(t)* | RFLP | RZ617, RGA-IR86 | 69.0 | IR64 | - |
| | *Pigd-1*(t) | RFLP | - | 11.3 | Sanhuangzan 2 | - |
| | *Pi55*(t) | SSR | H2, H66 | 100.6 | Yuejingsimiao 2 | |
| | | STS | | | Sanhuangzan 2 | |
| 9 | *PiI2*(t) | - | - | - | Ishikari Shiroke | Complete |
| | *Pi5*(t) | CAPS | 94A20r, 76B14f, 40N23r | 31.3–33.0 | RIL125, RIL249, | Complete |
| | | SNP | JJ817 | | RIL260 | |
| | | | | | (Moroberekan) | |
| | *Pi3*(t) | - | - | 31.3–33.0 | Pai-Kan-Tao | Complete |
| | *Pi15* | RAPD | BAPI15486, , BAPI15782, BAPil5844 | 31.3–34.9 | GA25 | Complete |
| | *Pi56*(t) | SSR | RM24022 | 31.3 | SHZ-2, BC-10 × | Complete |
| | | | | | TXZ-13 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | *Pihk2* | SSR | RM24048, RM24065, RM7390, RM3912, RM24019 | - | Heikezijing | Complete |
| 10 | *Pi28*(t) | RFLP | RZ500 | 114.7 | Azucena | - |
| | *Pigd-2*(t) | RFLP | R16 | 3.9 | | |
| 11 | *Pia* | CAPS | Yca72 | 36 | Aichi Asahi | Complete |
| | *Pi co39*(t) | *CAPS* | RGA8, RZ141, RGACO39 | 49.1 | Co39 | Complete |
| | *Pilm2* | RFLP | L457B, G2132b, RZ536, RG1109 | 56.2–117.9 | Lemont | Complete |
| | *Pi30*(t) | RFLP | OpZ11-f, RGA-IR14 | 59.4–60.4 | IR64 | - |
| | *Pi7*(t) | - | - | 71.4–84.3 | RIL29 (Moroberekan) | Complete |
| | *Pi34* | RFLP | C1172, E2021 | 79.1–91.4 | Chubu 32 | Partial |
| | *Pi38* | SSR | RM206, RM21 | 79.1–88.7 | Tadukan | - |
| | *Pif* | - | - | - | St No.1 | Partial |
| | *Pb1* | - | - | 85.7–91.4 | Modan | Partial |
| | *Pi44*(t) | *AFLP* | AF348, AF349 | 91.4–117.9 | RIL29 (Moroberekan) | Complete |
| | *Pik-h/Pi54* | SSR | RM206, RM144, RM224, RM1233 | 101.9 | Tetep, Taipei 309 | Complete |
| | *Pi1* | SNP | CRG11-7, K28 | 112.1–117.9 | C101LAC | - |
| | *Pi7*(t) | RFLP | RG103A, RG16 | 71.4–84.3 | RIL29 (Moroberekan) | Complete |
| | *Pik-m* | InDel | k6861, k2167 | 115.1–117.0 | Tsuyake | Complete |
| | | SSR | RM254, RM144 | | | |
| | | SNP | k641, k6441, k473, k7237 | | | |
| | *Pi18*(t) | RFLP | RZ536 | 117.9 | Suweon 365 | Complete |

| | | | | | | |
|---|---|---|---|---|---|---|
| | *Pik* | InDel | k6816, k2167 | 119.9–120.3 | Kusabue | Complete |
| | *Pik-p* | SNP | k641, k39575, k403, k3957 | 119.9–120.3 | HR22 | Complete |
| | *Pik-s* | SSR | RM144, RM224, RM1234 | 115.1–117.3 | Shin 2 | Complete |
| | *Pik-g* | - | - | - | GA20 | Complete |
| | *Pise1* | - | - | - | Sensho | |
| | *Pi-hk1* | SSR | RM27248, RM27318 | - | Heikezijing | Complete |
| | *Pikur2* | - | - | - | Kuroka | |
| | *Pi-1*(t) | SSR | RM12331, RM224 | 112.1–117.9 | ILs C10LAC and C101A5 | - |
| | *Pi47* | SSR | RM206, RM224 | | XZ3150 × CO39 | Complete |
| | *Pi49* | STS | K10, K134 | 1.01–1.89 | CO39 | |
| | *Pi60*(t) | inDel | K1-4, E12 | | 93-11 | Complete |
| | *Pi-jnw1* | SSR | RM27150, RM27381 | | Jiangnanwan | Complete |
| | | InDel | W26, W28, BS33, BS39, BS71 | | | |
| | *Pi65*(t) | SNP | SNP-2, SNP-8 | | | |
| | | InDel | InDel-1 | | Guangyu 129 | Complete |
| 12 | *Pi24* | RGA | RGA3 | 10.3 | Zhong 156 | Complete |
| | *Pi62*(t) | RLFP | RG869 | 12.2–26.0 | Yashiromochi | - |
| | *Pitq6* | RLFP | RG341a, RG869, L102, G1468a, RZ397, RZ257 | 29.2–47.5 | Teqing | Complete |
| | *Pi6*(t) | RLFP | RG869 | 32.6–63.2 | Apura | Complete |
| | *Pi12(*t) | - | - | 42.8–53.0 | Hongjiaozhan | Complete |
| | *Pi31*(t) | RFLP | O10-800 | 44.3 | IR64 | - |
| | *Pi32*(t) | RFLP | AF6 | 47.5 | IR64 | - |
| | *Ipi*(t) | RFLP | RG241X | 47.6–58.3 | BS125 × WL02 | - |

| | | | | | |
|---|---|---|---|---|---|
| *Ipi3*(t) | RFLP | RG241X | 47.6–58.3 | BS125 × WL02 | - |
| *Pi157* | - | - | 49.5–62.2 | Moroberecan | - |
| *Pita* | SNP | ta642, ta801, ta3, ta577, ta5, Pita440, Pita1042, Pita403 | 50.4 | Taducan | Complete |
| | RAPD | SP4B9, SP9F3 | | Yashiromochi | |
| *Pi39*(t) | CAPS | 39M6, 39M7 | 50.4 | Q15 | Complete |
| *Pi20*(t) | SSR | RM1337, RM5364, RM7102 | 51.5–51.8 | 1R24 | Complete |
| *Pgd-3*(t) | SSR | RM179 | 55.8 | Sanhuangzhan 2 | - |
| *Pi42(t)* | STS | STS5 | | DHR9 | - |
| | SSR | RRS44, RRS51, RRS60, RRS63, RRS6 | | | |
| *Pi4*(t) | RFLP | RG869, RZ397 | 47.5 | Apura | - |
| *Pita-2* | SNP | ta642, ta801, ta3, ta577 | 50.4 | Shimokita | Complete |
| *Pi19*(t) | - | - | | Aichi Asahi | Complete |
| *Pi21*(t) | RFLP | | 3.4–59.6 | Suweon 365 | |
| *Pi58*(t) | SSR | RM27954, RM27933, RM3103 | | Haoru × US-2 | |
| *Pi48* | SSR | RM5364, RM7102 | | XZ3150 × CO39 | Complete |
| *Pi61*(t) | Indel | M2, S29 | | 93-11 | Complete |

Ref:Damalas & Koutroubas, 2018; Koide *et al*., 2009. Chr, Chromosome; SSR, Simple sequence repeat; CAPS, Cleaved amplified polymorphism sequence; SNP, Single nucleotide polymorphism; RFLP, Restriction fragment length polymorphism; STS, Sequence tagged site; RAPD, Random amplified polymorphism DNA; RGA, Resistance gene analogue; AFLP, Amplified fragment length polymorphism.

#### 1.2.5.7 Integrated rice blast management

The success of rice blast control is dependent on the management strategy employed. However, every rice blast management strategy has its strength and weaknesses and there is therefore a need to integrate different management practices in a complementary manner. Deployment of rice blast management should be based on scientific information and available resources (Mew *et al.,* 2016).

## 1.3 Plant disease resistance mechanisms

Plants depend on innate immunity to defend themselves against plant pathogens, as described earlier, by deploying pathogen-associated molecular patterns (PAMPs) immunity and effector-triggered immunity (ETI) (Jones & Dangl, 2006). PAMPs are recognised by pattern recognition receptors (PRRs) of the host plant localised in the plant cell membrane (Katagiri & Tsuda, 2010). This activates PAMP-triggered immunity (PTI) that stops further colonization by the pathogen (Jones and Dangl, 2006). However, pathogens are able to overcome PTI by deploying effector proteins, localised inside the plant cell (Katagiri & Tsuda, 2010). This results in effector-triggered susceptibility (ETS) and subsequent colonization of the host plant. The effectors function by suppressing immunity (Hogenhout et al., 2009) or modulating metabolism (Djamei *et al.*, 2011) or preventing recognition of the invading pathogen (Mentlak *et al*., 2012). PTI is a weak immune response that has a slow signalling response and hence is vulnerable to pathogen effector-mediated perturbation (Katagiri & Tsuda, 2010). To protect themselves from pathogen effectors, plants depend on nucleotide-binding leucine rich repeat (NB-LRR) that recognise pathogen effectors and to trigger effector-triggered immunity (ETI). This is a stronger response than the PTI and leads to hypersensitive cell death (HR) that halts further colonisation of the

host (Greenberg, 1997). Race or cultivar specific resistance is determined by complementary pairs of dominant pathogen- encoded avirulence genes (*AVR*) and dominant plant resistance (R) genes (Nürnberger & Scheel, 2001).

### 1.3.1 Rice innate immunity against *Magnaporthe oryzae*

Plants are capable of receiving and responding to endogenous and exogenous signals. Endogenous signals arise from stressed, damaged or malfunctioning cells whilst exogenous signals arise from PAMPS, toxins, enzymes, effectors and toxic pollutants. Plant receptors are located at the cell surface or within the cytoplasm and have affinity to even very low concentration of ligands, thus enabling plants to develop an effective surveillance system against pathogens (Tör *et al.*, 2009). The domain organisation of plant receptors is shown in Figure 1-3. There are three types of membrane bound PRRs in plants: (i) receptor-like kinases (RLKs) that contain extracellular domains such as leucine rich repeats (LRRs), lectin, lysine motifs (LysM) or wall-associated kinases. They also contain a transmembrane domain and an intracellular kinase domain (ii) receptor-like proteins (RLPs) that consist an extracellular LRR domain and C-terminal membrane anchor. Unlike the RLKs, they lack an intracellular kinase domain (iii) polygalacturanase inhibiting proteins (PGIP) that only consist of an LRR domain. The extracellular PRRs associated with rice immunity are shown in Table 1-3.

Intracellular plant PRRs consist of a nucleotide binding domain and leucine-rich repeats (NB-LRR) and are encoded by plant resistance (R) genes (Tör *et al.*, 2009). NB-LRRs associated with rice immunity are shown in Table 1-4.

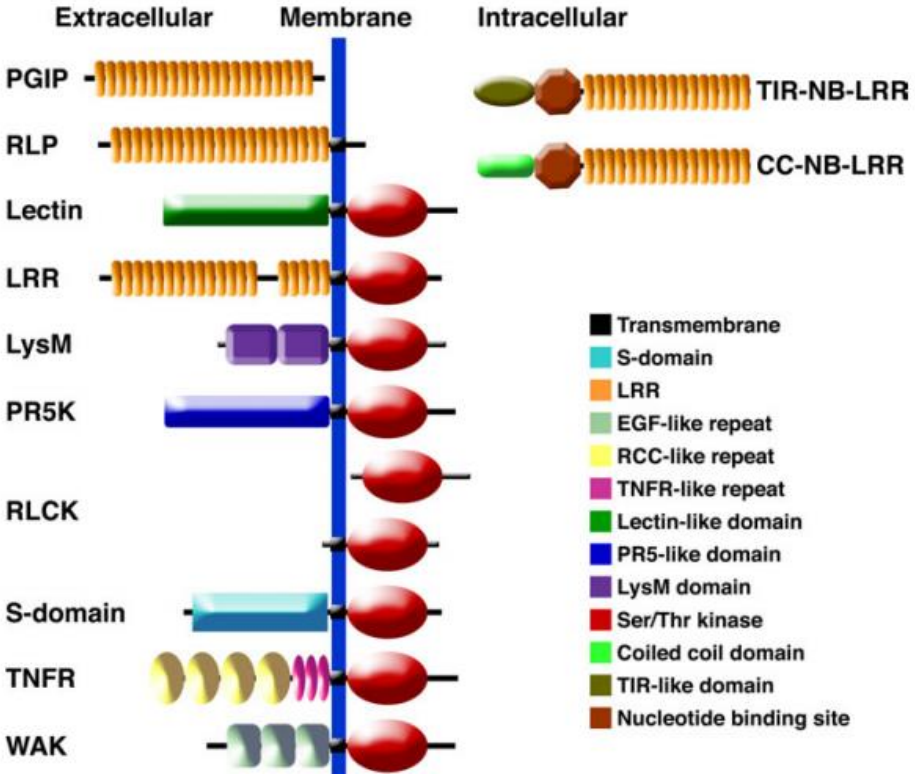


Figure 1-3. Domain organisation of typical extracellular and intracellular plant receptors. Receptor proteins provides a surveillance mechanism by which plants utilise to detect signals such as molecular associated pattern molecules (MAMP) from the invading pathogens. Extracellular PGIP, RLP and RLK-type directly or indirectly recognises signals from the invading pathogen. Subsequent heterodimerization, phosphorylation and conformational changes promote interactions with regulatory elements such as RLCKs to spread the message within the cell. NB-LRR proteins recognise directly or indirectly pathogen-specific signals, such as effector molecules, invoking a signalling cascade that triggers resistance against pathogens. Abbreviations: PGIP, polygalacturonase inhibitor protein; RLP, receptor-like proteins; RLK, receptor-like kinase; S-domain, self-incompatible domain; TNFR, tumour necrosis factor receptor; WAK, wall associated kinase; NB, nucleotide binding; TIR, Toll and Interleukin 1 transmembrane receptor; CC, coiled-coil; EGF, epidermal growth factor; RCC, regulator of chromosome condensation; Ser/Thr, serine/threonine (Tör *et al*., 2009).

Fritz-Laylin *et al.* (2005) identified 90 RLPs in rice and compared them with characterised RLPs from Arabidopsis (*Arabidopsis thaliana*) and other plants. The analyses indicated that rice RLPs cluster into four super clades three of which comprise of RLPs known to be involved in plant defence. The study further identified 73 candidate R genes in rice.

In rice, two lysin motif (LysM)-containing plasma membrane proteins, chitin elicitor-binding protein (CEBiP) and chitin elicitor receptor kinase 1 (OsCERK1), are important in the recognition of chitin. CEBiP and OsCERK1 consists of two and three extracellular LysM domains respectively. In addition to the extracellular domain, OsCERK1 consists of an intracellular Ser/Thr-kinase domain. A knockdown of either CEBiP or OsCERK1 impaired chitin-induced defence in rice indicating that both of these proteins are required for chitin signalling in rice. osCEBiP and OsCERK1 undergo hetero-oligomerization indicating that the two proteins work cooperatively to regulate the chitin response by formation of a complex receptor (Shimizu *et al.,* 2010). This is contrary to the chitin-induced defence response in *Arabidopsis* that only requires AtCERK1, a homologue of OsCERK1 (Shinya *et al.,* 2012). The OsCEBiP and OsCERK1 hetero-oligomer in rice phosphorylates a member of the receptor-like cytoplasmic kinases (RLCKs), OsRLCK185, initiating several chitin- and peptidoglycan-triggered signalling events, such as the ROS burst, MAP kinase activation and defence gene expression (Yamaguchi *et al.,* 2013). In addition, LysM–containing proteins, LYP4 and LYP6, have been shown to have dual function of sensing chitin and bacterial peptidoglycan (PGN) and silencing of either of the *LYP* gene impaired PGN- or chitin-induced defence response in rice. This was associated with reduced defence-related activities, including reactive oxygen species generation, defence gene activation, and callose deposition, leading to reduced resistance against the bacterial pathogen *Xanthomonas oryzae* pv. o*ryzae* and *M. oryzae (Liu et al.,* 2012).

The rice RLK gene *Xa21* confers broad spectrum to *X. oryzae* and was one of the first receptors to be isolated and characterised. Several *Rax* genes (required for activation of *Xa21*) have since been identified and are located in a single

operon (*raxSTAB*) that includes a tyrosine sulfotransferase (RaxST) and three components (*RaxA, RaxB,* and *RaxC*) of a predicted type 1 secretion system. It has been suggested that a tyrosine-sulfated, type 1-secreted protein activates Xa21-mediated immunity (Chen & Ronald, 2011).

The flagellum of bacteria is composed of flagellin and provides an immune response trigger in both plants and animals. In plants, a conserved flg22 epitope triggers immunity in *Arabidopsis* that carries a FLS PRR receptor. OsFLS2 is the rice orthologue of *Arabidopsis* FLS2 and is involved in mediating resistance against bacterial pathogens. It has been shown that flagellin from incompatible strains of *Pseudomonas avenae* and *Acidovorax avenae* triggered an immune response in rice cells that was associated with $H_2O_2$ generation, hypersensitive cell death and PR gene expression. It has therefore been suggested that flg22 signalling pathways are conserved between *Arabidopsis* and rice (Tanaka *et al.,* 2003; Chen *et al.*, 2000).

Plant Rac/Rop small GTPases constitute a subfamily of the Rho family of small GTPases and are involved in various signal transduction activities, including defence responses, pollen tube growth, root hair development, reactive oxygen species (ROS) generation and hormone responses in plants (Chen & Ronald, 2011). In rice, there are seven genes, *OsRac1–OsRac7*, with *OsRac1* being involved in plant defence related activities and required for both the ETI and PTI immune response (Chen & Ronald, 2011). Rice *OsRac1* is involved in ROS generation and cell death and is known to regulate ROS and lignin production by interacting with NADPH oxidase and cinnamoyl-CoA reductase (CCR) respectively (Kawasaki *et al.*, 1999; Kawasaki *et al.*, 2005).

Table 1-3. Pattern recognition receptors and co-receptors associated with rice immunity

| PRR | Protein structure | Function |
|---|---|---|
| CEBiP | LysM RLP | Chitin receptor |
| LYP4 | LysM RLP | Chitin and PGN receptor |
| LYP6 | LysM RLP | Chitin and PGN receptor |
| OsFLS2 | LRR RLK | Recognizes flg22 and triggers immunity |
| XA21 | LRR RLK | Recognizes RaxX21-sY and triggers immunity |
| OsCERK1 | LRR RLK | Co-receptor of CEBiP, LYP4 and LYP6 |
| OsRLCK185 | Receptor-like cytoplasmic kinases | Interacts with OsCERK1 and important for chitin- and PGN-induced immunity |
| OsRLCK176 | Receptor-like cytoplasmic kinases | Interacts with OsCERK1 and important for chitin- and PGN-induced immunity |
| OsSERK1 | LRR RLK | Regulates BR-mediated development signalling |
| OsSERK2 | LRR RLK | Co-receptor kinases of XA21 and regulates BR-mediated development signalling |

Source: Tör *et al.* (2009).

Table 1-4. Cloned rice resistance genes (R) and known avirulence genes of *M. oryzae* (*AVR*) and *X. oryzae* pv. *oryzae*

| **R gene** | **Protein structure** | ***AVR*** | **Encoded protein** | **Pathogen** |
|---|---|---|---|---|
| *Pib* | NB-LRR | *AVR-PIB* | 75 AA secreted protein | *M. oryzae* |
| *Pita* | NB-LRR | *AVR-PITA* | 224 AA secreted | *M. oryzae* |
| *Pi9* | NB-LRR | *AVR-PI9* | 91 AA secreted protein | *M. oryzae* |
| *Piz-t* | NB-LRR | *AVR-PIZ-T* | 108 AA secreted protein | *M. oryzae* |
| *Pil*$^{C}$ | | *AVR-PII* | 70 AA secreted protein | *M. oryzae* |
| *Pi2* | NB-LRR | ND | | *M. oryzae* |
| *Pi36* | NB-LRR | ND | | |
| *Pi-d2* | B lectin RLK | ND | | |
| *Pi33*$^{c}$ | | *ACE1* | Polyketide synthase | |
| *Pi37* | NB-LRR | ND | | |
| *Pi50*$^{a}$ | NB-LRR | ND | | |
| *Pi64* | NB-LRR | ND | | |
| *Pikm*$^{a}$ | NB-LRR | *AVR-PIK/KM/KP* | 113 AA secreted protein, five alleles (A–E) | |
| *Pit* | NB-LRR | ND | | |
| *Pi5*$^{a}$ | NB-LRR | ND | | |

| | | | | |
|---|---|---|---|---|
| *Pid3* | NB-LRR | ND | | |
| *Pid3-a4* | NB-LRR | ND | | |
| *Pi54* | NB-LRR | ND | | |
| *Pish* | NB-LRR | ND | | |
| *Pik* | NB-LRR | *AVR-PIK/KM/KP* | 113 AA secreted protein, five alleles (A–E) | |
| *Pikp* | NB-LRR | *AVR-PIK/KM/KP* | 113 AA secreted protein, five alleles (A–E) | |
| *Pia*[a,b] | NB-LRR | *AVR-PIA* | 85 AA secreted protein | |
| *Pi-co39*[a,b] | NB-LRR | | 89 AA secreted protein | |
| *Pi25* | NB-LRR | | - | |
| *Pi1* | NB-LRR | | - | |
| *Pi21* | Proline-containing protein | ND | -- | |
| *Pb1* | NB-LRR | ND | -- | |
| ND | | *PWL2* | 145 AA secreted protein | |
| *Xa5* | TFIIA transcription factor | *AVRXA5/PTHXO7* | | *X. oryzae* |
| *Xa13* | MtN3/saliva domain protein | *AVRXA13/PTHXO1* | TALE | *X. oryzae* |
| *Xa25* | MtN3/saliva domain protein | ND | | *X. oryzae* |
| *Xa3/Xa26* | LRR-RLK | *AVRXA3* | TALE | *X. oryzae* |
| *Xa27* | Rice unique gene | *AVRXA27* | TALE | *X. oryzae* |

| | | | | |
|---|---|---|---|---|
| *Xa1* | NB-LRR | ND | | *X. oryzae* |
| *Os11n3* | NB-LRR | *AVRXA7* | TALE | *X. oryzae* |
| *Xa10* | Executor R protein, encodes 126 AA, with four potential transmembrane helices | *AVRXA10* | TALE | |
| *Xa23* | Executor R protein, encodes 113 AA, with four potential transmembrane helices | *AVRXA23* | TALE | |
| *Rxo1*[d] | NB-LRR | *AVRRXO1* | - | *X. oryzae* |
| *Stv11* | Sulfotransferase | ND | - | Rice stripe virus |

Source: Liu & Wang 2016.

[a]The function of these three R genes requires two NB-LRR members.
[b]These two R genes share the same NB-LRR gene locus.
[c]The gene has not been cloned yet.
[d]This gene was cloned from maize.
ND=not determined.

The most effective way of managing rice blast disease is by deployment of cultivars that are durably resistant. Gene pyramiding offers one strategy that can be employed to develop durable rice blast resistant cultivars (Fukuoka *et al.,* 2015; Koide *et al*., 2010). This involves introgressing more than one resistance gene into a commercial rice cultivar to offer resistance to a broad spectrum of pathotypes. However, to ensure that the resistance genes are stacked into the commercial varieties effectively, it is vital to understand the population structure of the pathogen and, in particular, the most prevalent pathotypes within a given region. To address the problem of rice blast in sub-Saharan Africa (SSA), a project was initiated to define the genetic and phenotypic diversity of the rice blast fungus population from SSA. A major research project funded by Biotechnology and Biological Sciences Research Council (BBSRC), the Bill and Melinda Gates Foundation, the Halpin rice blast research scholarship and the United Kingdom Department for International Development (DFID) was established in 2012, co-ordinated by Prof. Nick Talbot at the University of Exeter. The project aimed to define the pathogen population biology, identify major resistance genes that could exclude the majority of the indigenous pathogen population, and use the information to introgress durable rice blast resistance to commercially grown rice varieties using marker assisted breeding strategy.

My research project aimed to meet one of the main objectives of this project. The goal of this study is to define the population structure of the Kenyan rice blast population by determining genotypic and phenotypic diversity within the Kenyan *M. oryzae* population. The specific objectives of my PhD project were:

1. To collect a representative population of *M. oryzae* from all of the rice-growing regions of Kenya.

2. To determine the virulence diversity of Kenyan *M. oryzae* isolates on a set of rice blast differential lines.

3. To determine genotypic diversity of Kenyan *M. oryzae* isolates by analysis of the internal transcribed spacer region sequences (ITS), DNA fingerprinting with a DNA repetitive element, Pot2, and by whole genome sequencing.

4. To identify and characterise effector-encoding genes that define *M. oryzae* isolates from Kenya.

# 2 Chapter 2: Materials and Methods

## 2.1 Collection and maintenance of *M. oryzae* and monoconidia culture stocks

Leaves and stalks of rice plants showing symptoms of rice blast infection (Figure 2-1) were collected from rice growing regions in Kenya viz. Coastal, Western and Central regions of Kenya during two study visits in 2014 and 2015 (Figure 2-2-2.5). These areas represent different rice production systems and agro-ecological zones (Table 2-1). *M. oryzae* was isolated from infected leaves using the moist chamber method (Correll *et al*., 2009). Diseased leaves were incubated for 24 h in a Petri dish lined with moist sterile filter paper. The lesions were examined under a dissecting microscope and conidia picked from sporulating tissues were streaked on 2% (w/v) water agar using a sterile wire loop. The germinating conidia were picked and transferred onto rice bran agar medium (20 g rice bran, 20 g agar $L^{-1}$) (Correll *et al*., 2009). To enable long-term storage, *M. oryzae* was grown through sterile filter paper discs (3 mm, Whatman International), which were then desiccated and stored at -20$^{o}$C. The fungus was routinely incubated in a controlled temperature room at 24$^{o}$C with a 12-h light/12-h dark cycle. A total of 290 isolates were collected (Appendix 1).

Figure 2-1. Supa rice variety grown in coastal Kenya showing severe leaf necrosis from rice blast infection

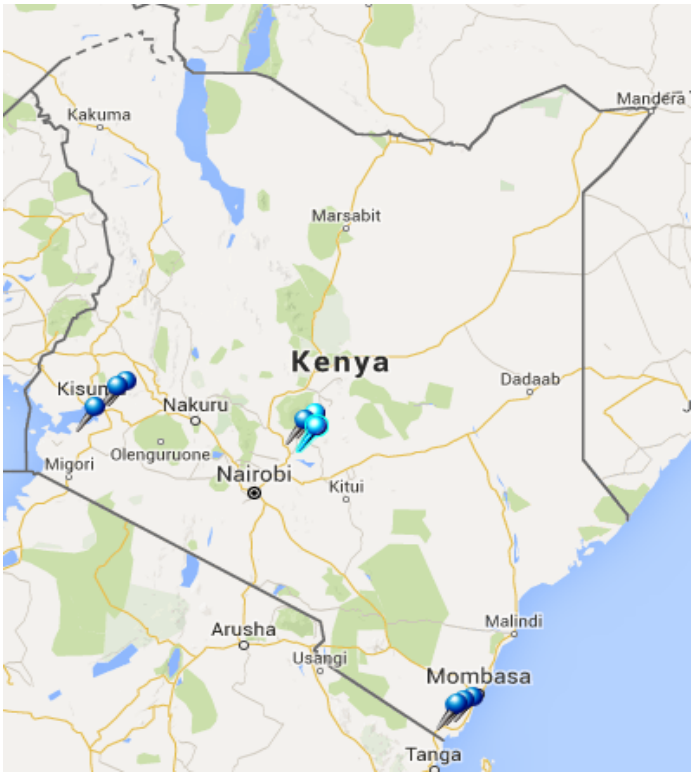


Figure 2-2. Map of Kenya showing rice blast collection sites. The diseased leaf samples were collected from the main rice-growing regions in Kenya and these include coastal, central and western Kenya

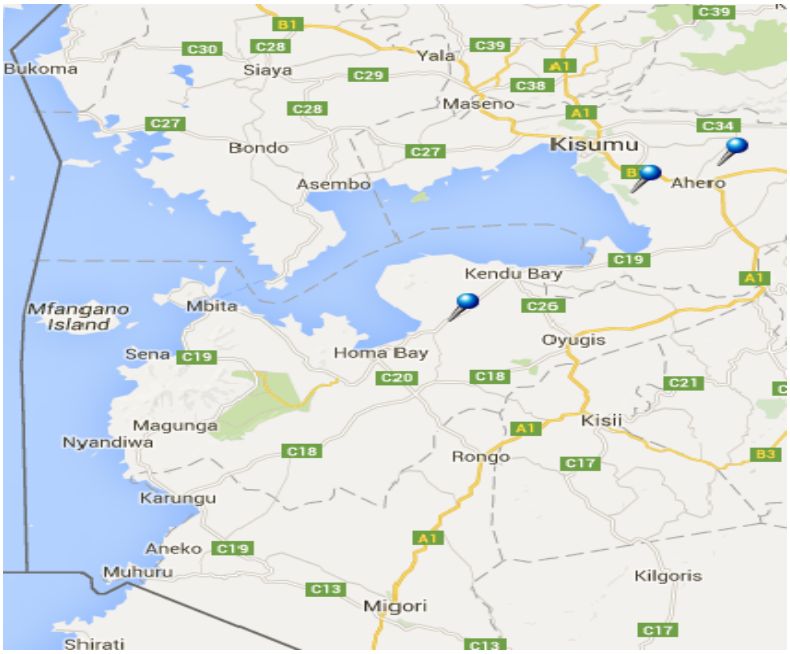


Figure 2-3. Map of western Kenya showing rice blast collection sites in Ahero and Homa-Bay

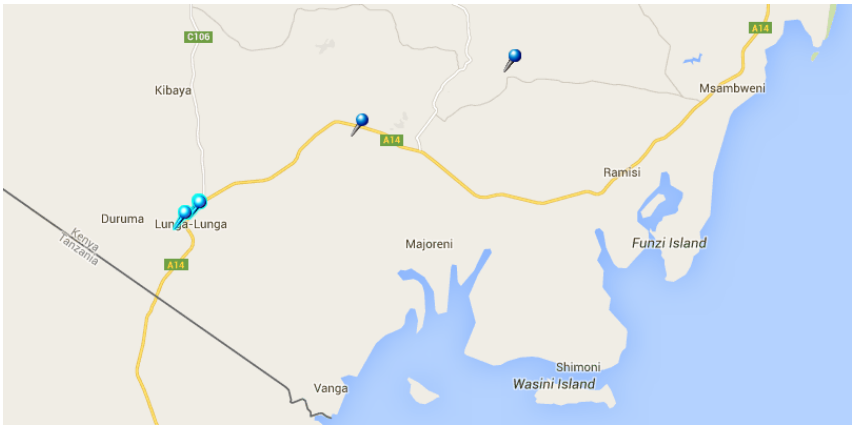


Figure 2-4. Map of coastal Kenya showing rice blast collection sites

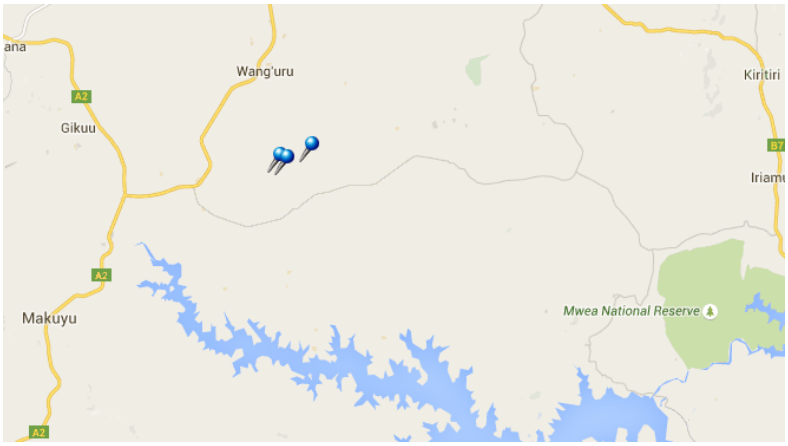


Figure 2-5. Map of central Kenya rice blast collection sites

Table 2-1. Characteristics of rice blast collection sites in Kenya

| Region | Agro-ecological zone | Rice Production system | Main rice varieties grown | No. of farms in collection site |
|---|---|---|---|---|
| Coastal | Coastal lowland | Rain-fed | Supa, Durado precoce, Sindano, local landraces | 4 |
| Western | Lower midland | Irrigated and rainfed | ITA 310, Nerica 1, Nerica 4, Nerica 10, Nerica 11, IR 2793, Sindano | 6 |
| Central | Lower Midland | Irrigated | Basmati 310, Basmati 217, BW 196 | 7 |

## 2.2 Pathotype analysis

Pathotype analysis was carried out using rice blast differential lines, described in section 4.2. Rice seeds were first surface sterilized with sodium hypochlorite, 8% (w/v) for 2 min and washed 3 times with sterile $dH_2O$. The seeds were pre-germinated for 2-3 days in Petri dishes lined with sterile moist filter paper. The germinating seeds were transplanted into plastic trays, 25 cm x 35 cm, containing compost (John Innes No. 2). For each monogenic line, 8 seedlings were sown and each tray comprised a complete set of the rice blast differential lines. Rice seedlings were grown for 21 days and then inoculated with *M. oryzae*. To prepare the inoculum, conidia from a 14-day-old plate culture were harvested in $dH_2O$. The spores were filtered through sterile Miracloth (Calbiochem) and subjected to centrifugation at 5,000 *x g.* The pellet was resuspended in 0.2% gelatin (BDH) to a final concentration of $5 \times 10^4$ conidia $ml^{-1}$. The suspension, 10 ml, was spray-inoculated onto the rice plants using an artist's airbrush (Badger Airbrush, Franklin Park, Illinois, USA). After spray-inoculation, the trays were placed into plastic storage boxes and grown in a controlled environment chamber (REFTECH, Holland) at 24°C with a 12-h light/12-h dark cycle and 80% relative humidity (Valent & Chumley, 1991). The trays were covered for the first 48 h to

maintain high humidity. Disease assessment was carried out 6-7 days post inoculation using a quantitative disease scale of 0-5. Disease scores of 0-2 were rated as resistant reactions, while scores 3-5 were rated as susceptible reactions (Table 2-2).

Table 2-2. Qualitative disease assessment scale for rice blast

| Score | Lesion type |
|---|---|
| 0 | No evidence of infection. |
| 1 | Brown specks smaller than 0.5 mm in diameter, no sporulation. |
| 2 | Brown specks about 0.5-1.00 mm in diameter, no sporulation.<br>Small lesions with distinct tan centres surrounded by a darker brown margin approximately 1mm in diameter. |
| 3 | Roundish to elliptical lesion about 1-3 mm in diameter with grey centre surrounded by brown margins, lesions capable of sporulation.<br>Small eyespot lesions less than one and a half times the interval between thin veins or less than 1.5 mm in diameter surrounded by dark brown, lesions capable of sporulation. |
| 4 | Typical spindle shaped blast lesion capable of sporulation, 3 mm or longer with necrotic grey centres and water soaked brown margins and little or no coalescence of lesion.<br>Intermediate size eyespot lesions less than twice the interval between thin veins or less than 2 mm in diameter. |
| 5 | Lesions as in 4 but about half of one or two leaf blades killed by coalescence of lesion. Large eyespot lesions more than twice the interval between thin veins or more than 2 mm in diameter. |

## 2.3 Tolerance of Kenyan varieties to Kenyan *M. oryzae* isolates

Rice varieties commonly grown in Kenya were evaluated for tolerance to rice blast. These varieties include Basmati 370, BW 196, IR2793-80-1, ITA 310, and Durado precoce, Nerica 1, Nerica 4, Nerica 10 and Nerica 11. LTH was used as a susceptible control. The seeds were surface sterilised and pre-germinated as previously described. The germinating seed were transplanted into plastic trays size 25 cm x 35 cm containing compost (John Innes No.2). The seedlings were

planted in a completely randomised design with three replicates. Inoculum preparation and infection was performed as described previously. Disease assessment was carried out 6-7 days post-inoculation using a quantitative disease scale of 0-9. Disease scores of 0-3 were rated as resistant reactions, while scores 4-9 were rated as susceptible reactions (Table 2-3).

Table 2-3. Quantitative disease assessment scale for rice blast

| Score | Lesion type and severity |
|---|---|
| 0 | No lesions observed |
| 1 | Small brown specks of pin-point size or larger brown specks without sporulating centres |
| 2 | Small roundish to slightly elongated, necrotic grey spots, about 1-2 mm in diameter, with a distinct brown margin |
| 3 | Lesion type is the same as in scale 2, but a significant number of lesions are on the upper leaves |
| 4 | Typical susceptible blast lesions 3 mm or longer, infecting less than 4% of the leaf area |
| 5 | Typical blast lesions infecting 4-10% of the leaf area |
| 6 | Typical blast lesions infection 11-25% of the leaf area |
| 7 | Typical blast lesions infection 26-50% of the leaf area |
| 8 | Typical blast lesions infection 51-75% of the leaf area and many leaves are dead |
| 9 | More than 75% leaf area affected |

Analyses of variance (ANOVA) for the disease scores was performed using Stata ver 14. Means with significant differences were separated by the Bonferroni test at 0.05 significance level.

## 2.4 Nucleic acid analysis

### 2.4.1 DNA Extraction

Filter paper disks colonized with *M. oryzae* were regenerated on complete medium (CM) (10 g $L^{-1}$ glucose, 2 g $L^{-1}$ peptone, 1 g $L^{-1}$ yeast extract (BD Biosciences), 1 g $L^{-1}$ casamino acids, trace elements (22 mg $L^{-1}$ zinc sulfate heptahydrate, 11 mg $L^{-1}$ boric acid, 5 mg $L^{-1}$ manganese(II) chloride tetrahydrate, 5 mg $L^{-1}$ iron(II) sulfate heptahydrate, 1.7 mg $L^{-1}$ cobalt(II) chloride hexahydrate, 1.6 mg $L^{-1}$ copper(II) sulfate pentahydrate, 1.5 mg $L^{-1}$ sodium molybdate dehydrate, 50 mg $L^{-1}$ ethylenediaminetetraacetic acid,), vitamin supplement (100 µg $L^{-1}$ biotin, 100 µg $L^{-1}$ pyridoxine, 100 µg $L^{-1}$ thiamine, 100 µg $L^{-1}$ riboflavin, 100 µg $L^{-1}$ p-aminobenzoic acid, 100 µg $L^{-1}$ nicotinic acid), nitrate salts (6 g $L^{-1}$ sodium nitrate, 0.5 g $L^{-1}$ potassium chloride, 0.5 g $L^{-1}$ magnesium sulfate heptahydrate, 1.5 g $L^{-1}$ potassium dihydrogen phosphate), pH to 6.5, 15 g $L^{-1}$ agar,) (Talbot *et al*., 1993). All chemicals were obtained from Sigma unless otherwise stated. A sterile cellophane disc (Lakeland) was placed onto the plate before inoculation. Once the mycelium had grown across the cellophane disc, it was peeled from the medium, placed in aluminium foil and snap frozen in liquid nitrogen. Genomic DNA was extracted using the CTAB (Cetyltrimethylammonium Bromide) method, as described in Talbot *et al*., 1993. Briefly, the mycelium was ground in liquid nitrogen and transferred into 1.5 µl microcentrifuge tubes and 500 µl of 2x CTAB extraction buffer (20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2x CTAB) was added. The samples were incubated at 65$^{o}$C for 30 min with occasional shaking and then 500 µl of chloroform: isoamyl alcohol (24:1 v/v) was added. The samples were then shaken vigorously for 20 min at room temperature. Following centrifugation at 13,000 *x g* for 10 min, the supernatant was removed and transferred into a fresh tube. The chloroform: isoamyl alcohol extraction step was

repeated and the supernatant carefully removed and transferred into a fresh tube containing 1 ml of isopropanol. DNA was precipitated by overnight storage at -20$^{o}$C. Centrifugation was carried out at 13,000 *x g* for 10 min and the supernatant discarded. The pellet of nucleic acid was air dried at room temperature and re-suspended in 500 µl sterile d$H_2O$ before adding 50 µl of 3 M sodium acetate (NaOAc) and 1 ml of ice-cold 100% ethanol. The samples were incubated at -20$^{o}$C for 10 min and then subjected to centrifugation at 13,000 *x g* for 20 min. The supernatant was discarded and the pellet was washed with 400 µl of ice-cold 70% (v/v) ethanol. The purified nucleic acid fraction was recovered by centrifugation at 13 000 *x g* for 5 min. The pellet was air dried at room temperature and resuspended in 50 µl sterile $H_2O$ containing RNase A (10 µg $ml^{-1}$). Genomic DNA samples were routinely stored at -20$^{o}$C.

### 2.4.2 DNA manipulations

#### 2.4.2.1 Digestion of genomic DNA by restriction enzymes

Restriction endonucleases were routinely obtained from Promega UK Ltd or New England Biolabs (Hitchin, UK). DNA digestion was carried out using the appropriate buffer solution provided by the manufacturer, 0.2-1 µg DNA and 5-10 units of enzyme in a total volume of 50 µl. Reactions were incubated at the optimum temperature for 4 h.

#### 2.4.2.2 DNA gel electrophoresis

Digested DNA and PCR amplification products were fractionated by gel electrophoresis in 0.8 % (w/v) agarose gel in 1X Tris-borate EDTA (TBE) buffer (0.09 M Tris-borate, and 2 mM ethylenediaminetetraacetic acid). To visualise the DNA, ethidium bromide was added to molten agarose gel to a final concentration of 0.5 µg $ml^{-1}$. A size marker, 1 kb plus, (Invitrogen) was used to estimate the size

of DNA products. Fractionated DNA was visualised and recorded using a Bio-Doc-ITTM gel documentation system and the image was printed using a Sony hybrid graphic printer UP-X898MD.

#### 2.4.2.3 Amplification of DNA by Polymerase Chain Reaction (PCR)

DNA fragments were routinely amplified by Polymerase Chain Reaction (PCR). Unless otherwise stated, PCR reactions were performed as follows: 12.5 µl GoTaq® G2 Green Master Mix (Promega, UK), 50-100 ng of template DNA, 0.5 µM each of forward and reverse primers and nuclease-free water to a total volume of 25 µl. PCR amplification was carried out using an Applied Biosystems, Veriti thermal cycler ®. An initial denaturation step at 94$^{o}$C for 5 min, was followed by 35 cycles of PCR cycling parameters: 94$^{o}$C for 30 s, 56-62$^{o}$C for 30 s, and 72$^{o}$C for 1 min per 1 kb of expected product length, followed by a final extension at 72$^{o}$C for 10 min.

For experiments requiring high fidelity (effector cloning experiments), Phusion high-fidelity DNA polymerase (New England Biolabs (Hitchin, UK)) was used. PCR reactions were performed as follows: 10 µl of 5X Phusion HF buffer, dNTPs (200 µM), forward and reverse primers (0.5 µM of each) 50-100 ng of template DNA, 1 unit of Phusion DNA polymerase and $H_2O$ to a final volume of 50 µl. The following PCR conditions were used: Initial denaturation at 98°C for 30 s, and 35 cycles of PCR cycling parameters: 98$^{o}$C for 10 s, 58-60$^{o}$C for 30 s, and 72$^{o}$C for 30 s per 1 kb of desired length of PCR product, followed by a final extension at 72$^{o}$C for 10 min.

SapphireAmp® Fast PCR Master Mix (Clontech) was used for colony PCR screening. Each reaction contained 2X Premix SapphireAmp® Fast PCR Master Mix, 0.2 µM each of forward and reverse primer, 100 pg to 10 ng template DNA

and $dH_2O$ to a final volume of 50 µl. The following PCR conditions were used: an initial denaturation step at 94°C for 1 min was followed by 35 cycles of PCR cycling parameters: 98°C for 5 s, 55°C for 5 s and 72°C for 10 s per 1 kb of desired length of PCR product length. Amplicons were gel-purified as described below.

#### 2.4.2.4 Gel purification of DNA fragments

DNA was purified from agarose gel using the Wizard Plus SV Gel and PCR Clean-up System® kit (Promega, UK), according to the manufacturer's instructions. The DNA fragment was removed from the gel using a razor and placed in a pre-weighed vial. The mass of the gel was determined and Membrane binding solution (4.5 M guanidine isothiocyanate and 0.5 M potassium acetate, pH 5.0) was added to the gel slice at a ratio of 10 µl per 10 mg of gel slice. Samples were incubated at 65°C and mixed regularly until the gel was completely dissolved. The dissolved sample was transferred into a Wizard® SV Minicolumn inserted in a 2 ml collection tube, and incubated at room temperature for 1 min to allow the DNA to bind to the column. Following centrifugation at 13,000 *x g* for 1 min, the flow-through was discarded and the column was washed with 500 µl Membrane wash solution. The sample was subjected to centrifugation for 1 min and the wash was repeated. Centrifugation was repeated for an additional 1 min and the column was transferred to a clean microtube. The bound DNA was eluted in 30 µl of nuclease-free water and recovered by centrifugation at 13,000 *x g* for 1 min. The DNA was stored at -20°C.

### 2.4.3 DNA Cloning of candidate effector gene

A DNA fragment of 3025 bp comprising the candidate effector gene was amplified from KE0002 genomic DNA with primers

Avrpiz5F4:

5’CGCGGTGGCGGCCGCTCTAGACCCAGTTGCCGTTCTTTCGCTTAC3’ and Avrpiz5R4:

5’CTATAGGGCGAATTGGGTACCGATAAGTTACGTGCGGGTCCATAC3’

designed to introduce Kpn1 and Xbal extensions in the PCR fragment. The extensions overlap with adjacent fragments allowing the ends to fuse during cloning. The DNA fragment was ligated in plasmid pCB 1532 and cloned using the In-fusion HD Cloning Kit (Clontech) as per the manufacturer’s instructions.

Cloning reactions were set up as follows: 2 µl of 5X In-fusion HD Enzyme Premix, 10-200 ng of the PCR fragment, 50-200 ng linearized vector, and $dH_2O$ to a total volume of 10 µl. The reaction was incubated for 15 min at 50$^oC$ and then placed on ice. A 2.5 µl aliquot of the reaction mixture was added to 50 µl of Stellar Competent Cells in an ice-cold round-bottom plastic tube, mixed gently, and incubated on ice for 30 min. The bacterial cells were then heat-shocked at 42$^oC$ for exactly 45 s, and then placed on ice for 2 min. Then 450 µl of pre-warmed SOC medium was added to the cells and they were incubated at 37$^oC$ for 1 h with gentle shaking. An aliquot of this bacterial culture was plated onto a Lysogeny Broth (LB) agar plate containing ampicillin and incubated at 37$^oC$ overnight. Positive clones were verified by colony PCR and sequence analysis (see section 2.4.5).

### 2.4.4 Plasmid DNA extraction

Plasmid DNA was obtained using a commercially available kit (Promega PureYield™ Plasmid Midiprep System, Promega) as per the manufacturer’s instructions. A single colony of the positive bacterial colony was picked and inoculated in 50-100 ml LB medium containing the appropriate antibiotic. The cells were incubated at 37$^oC$ overnight and then harvested by centrifugation for

10 min at 4,000 *x g*. The resulting pellet was re-suspended in 3 ml of cell re-suspension solution (50 mM Tris [pH 7.5], 10 mM EDTA, 100 µg $ml^{-1}$ RNase A) in an Oak Ridge centrifuge tube. 3 ml of cell lysis solution (0.2 M sodium hydroxide, 1% sodium dodecyl sulfate) was added to the tube, and the contents were mixed by inverting 5 times. The tube was incubated at room temperature for 3 min and then 5 ml of neutralization solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, [final pH 4.2]) was added and the contents were mixed by inverting 10 times. The tube was then subjected to centrifugation for 15 min at 10,000 *x g* at room temperature. A column stack was then assembled by placing a PureYield™ Clearing Column on top of a PureYield™ Binding Column. The column stack was placed onto a vacuum manifold and the bacterial lysate poured into the column. A vacuum was applied to the column stack until the liquid had passed through both the clearing and binding columns. The vacuum was released slowly and the clearing column was discarded. A 5 ml aliquot of endotoxin removal wash solution was added to the binding column and a vacuum applied allowing the solution to be drawn through the column. A 20 ml of column wash solution was added to the binding column and the solution drawn through the vacuum again. The binding column was removed from the vacuum manifold and transferred to a clean 50 ml tube ensuring that there was no ethanol contamination on the wall of the column. DNA was recovered from the binding column by adding 600 µl of nuclease-free $H_2O$ to the column and incubating for 2 min at room temperature, followed by centrifugation for 5 min at 4,000 *x g.* The DNA was transferred into a 1.5 µl microcentrifuge tube before being stored at -20$^{o}$C.

### 2.4.5 DNA sequence analysis

All DNA sequence analyses were carried out at Eurofins MWG Operon using cycle sequencing technology (dideoxy chain termination/ cycle sequencing) on ABI 3730XL sequencing machines.

Multiple sequence alignment was performed using ClustalW2 program and shaded using Boxshade version 3.21.

## 2.5 Transformation of *M. oryzae*

A 2.5 $cm^2$ section of mycelium of *M. oryzae* was cut from a CM plate and blended in 150 ml of complete medium. This was incubated at 24$^{o}$C with shaking for 48 h. The mycelium was harvested by filtration through sterile Miracloth and washed in sterile distilled water. The mycelia were transferred to 50 ml conical tubes and 40 ml OM buffer (1.2 M magnesium sulfate, 10 mM sodium phosphate (pH 5.8), Glucanex 5% was added. This was incubated at 30$^{o}$C with gentle (75 rpm) shaking, for 2 to 3 h. The protoplasts were transferred to a sterile polysulfonate Oak Ridge tube and overlaid with an equal volume cold ST buffer (0.6 M sucrose, 0.1 M Tris-HCl (pH 7). Protoplasts were recovered by centrifugation at 5000 *x g* for 15 min at 4$^{o}$C in a swinging bucket rotor (Beckman JS-13.1) in a Beckman J2.MC centrifuge. The protoplasts were recovered from the OM/ST interface and transferred into two or three sterile Oakridge tubes, and then filled with STC (1.2 M sucrose, 10 mM Tris-HCl, (pH7.5), 10 mM calcium chloride,). Pelleted protoplasts were recovered by centrifugation at 3000 *x g* for 10 min at 4$^{o}$C, in a swinging bucket rotor centrifuge. The wash was repeated and the protoplasts were re suspended in 1.0 ml of STC and the concentration estimated using a haemocytometer.

Transformation was performed by combining the protoplasts with 4-6 ug of plasmid DNA in a total volume of 150 µl of STC. The mixture was incubated at room temperature for 15 to 25 min and then 1.0 ml of PTC (60% PEG 4000, 10 mM Tris-HCl, (pH 7.5), 10 mM calcium chloride) was added (in 2 to 3 aliquots) and gently mixed by inversion. The protoplasts were incubated at room temperature for 15 to 20 min and then added to 125-150 ml molten (45$^{o}$C) BDCM bottom agar (1.7 g L $^{-1}$ yeast nitrogen base without amino acids and ammonium sulfate, (Difco), 2 g L $^{-1}$ ammonium nitrate, 1 g L $^{-1}$ asparagine, 10 g L $^{-1}$ glucose, 0.8 M sucrose, pH 6), gently mixed and poured into 5-6 Petri dishes. The plates were covered with aluminium foil and incubated for 16 h at 24$^{o}$C after which they were overlaid with BDCM top agar (BDCM without sucrose), containing sulfonylurea (chlorimuron ethyl) 300 µg ml$^{-1}$.

## 2.6 Genotypic analysis using the ribosomal RNA-encoding gene cluster and genome sequence

The Internal Transcribed Spacer region (ITS) of ribosomal DNA (rDNA) was amplified using primers ITS1, TCCGTAGGTGAACCTGCGG and ITS4, TCCTCCGCTTATTGATATGC, as described by White *et al*. (1990). The reactions were carried out in a 25 µl volume as described in section 2.4.2.3, with a denaturing step of 94$^{o}$C for 30 s, an annealing temperature of 55$^{o}$C and an extension step at 72$^{o}$C for 60 s The DNA sequence was analysed by Eurofins Genomics using the ITS1 primer.

A phylogenetic tree based on ITS sequences was constructed using phylogeny.fr platform (Dereeper *et al*., 2008). Multiple sequence alignment was carried out with 16 iterations on the program MUSCLE version 3.8.31 (Edgar, 2004). Columns containing gaps were removed from the alignment. The phylogenetic tree was constructed using Maximum Likelihood (Felsenstein, 1981) on PhyML

version 3.1/3.0 program using 100 bootstraps, number of substitution rate categories = 8, substitution model=GTR (Guindon *et al*., 2010).

Using phylogenetic analysis based on ITS sequence analysis, a sub-set of isolates were then selected for whole genome sequencing. Genome sequencing was carried out using Illumina next generation DNA sequencing. Single nucleotide polymorphisms (SNPs) were identified and a phylogenetic tree constructed. In addition to Kenyan isolates, other archived isolates were also used in SNP analysis. Genomic DNA of different isolates of *M. oryzae* were sequenced on the Illumina HiSeq 2500 generating 150 base paired-ends reads (Illumina, Inc.). After quality filtering using the fastq-mcf program from the ea-utils package (http://code.google.com/p/ea-utils/), reads were mapped to the *M. oryzae* 70-15 reference genome version 8 (Dean *et al*., 2005) using BWA (Li & Durbin, 2010). Bespoke perl scripts were used to discover SNPs (based on minimum read depth of 10 and minimum base identity of 95%). A bespoke perl script was used to construct a pseudosequence for each strain based on base calls at each SNP position. A maximum-likelihood phylogenetic tree was constructed from this pseudosequence using PhyML (Guindon *et al*., 2010) using 100 bootstraps, number of substitution rate categories = 8, substitution model = GTR.

Gene calling was undertaken using Maker (Campbell *et al.,* 2015) trained using RNA-seq data from Kenyan isolate KE0002. Predicted gene sets from each isolate were clustered with transcripts from 70-15 using Proteinortho (Lechner *et al*., 2011). Functional annotation of the clustered genes was undertaken using BLAST2GO (Conesa *et al.*, 2005), and Pfam database (Finn *et al.,* 2016).

Sequences coding for known *M. oryzae AVR* genes were searched from the genomic data of sequenced isolates using nucleotide Basic Local Alignment Search Tool (BLASTn).

Prediction of sequences coding for effector genes was undertaken in isolate KE0002. Amino acid sequences of predicted genes were scanned for a signal peptide using SignalP (Nielsen *et al.,* 1997) and those that had a signal peptide were analysed using EffectorP program to identify potential effectors (Sperschneider *et al.*, 2016).

### 2.7 Pot2 rep-PCR DNA fingerprinting experiments

Pot2 rep-PCR was performed according to the protocol described by George *et al.* (1998) with modifications using primers Pot2-1 (5′ CGGAAGCCCTAAA-GCTGTTT 3′) and Pot2-2 (5′ CCCTCATTCGTCACACGTTC 3′). DNA fragments were amplified using GoTaq® G2 Green Master Mix (Promega, UK) as described in section 2.4.2.3, with modifications to the parameters. An initial denaturation step was performed at 95$^{o}$C for 2.5 min, followed by four cycles of 94$^{o}$C for 1 min, 62$^{o}$C for 1 min and 65$^{o}$C for 10 min, and then 26 cycles of 94$^{o}$C for 30 s, 62$^{o}$C for 1 min and 65$^{o}$C for 10 min. The reaction was completed by a final extension of 15 min at 65$^{o}$C.

The gel images were processed and analysed using the GelJ ver. 2.0 software. Briefly, the images were loaded into the software and pre-processed by inverting the colours and adjusting the contrast/brightness. The lanes and bands were detected automatically and adjusted manually according to the software developer instructions. 1 kb plus DNA marker was used as the reference lane to normalise the images. Similarity matrix was generated by Dice coefficient and tree constructed using Unpaired Group Arithmetic Mean.

## 2.8 Determination of mating type distribution of Kenyan *M. oryzae* isolates

Mating type gene loci (*MAT1.1* and *MAT1.2*) of Kenyan *M. oryzae* isolates was determined using gene-specific primers previously described (Takan *et al.,* 2012)**.**

MAT1-1 F: 5'-TGCGAATGCCTACATCCTGTACCGC-3';
MAT1-1 R: 5'-CGCTTCTGA GGAACGCAGACGACC-3'
MAT1-2 F: 5'-TCTGCTTG AAGCTGCAATACAACGG-3'
MAT1-2 R: 5'-CAT GCGAGGGTGCCATGATAGGC-3'

# Chapter 3: Genetic diversity of Kenyan *Magnaporthe oryzae* isolates

## 3 Introduction

### 3.1 Internal transcribed spacer regions and their application in phylogenetic analysis

Molecular tools have been utilised to reliably evaluate biological diversity of organisms (Gherbawy & Voigt, 2010). There have been efforts by various multi-disciplinary teams of scientists to identify suitable DNA barcodes specific for each organism. In animals, a segment of the mitochondrial gene encoding the cytochrome c oxidase subunit 1 (CO1) has been proposed as a suitable DNA barcode (Pennisi, 2007). In plants, a number of loci have been identified as suitable DNA barcodes. However, the Consortium for the Barcode of Life (CBOL) plant working group has proposed plastid genes, *matK* and *rbcL* as the most promising plant DNA barcodes (Hollingsworth *et al.,* 2009). In fungi, Schoch and colleagues (Schoch *et al.,* 2012) evaluated the potential of ribosomal regions and protein-coding genes as potential DNA barcodes for fungi. They proposed that the internal transcribed spacer (ITS) region of the ribosomal RNA-encoding gene unit as having the highest probability for successful identification of most fungal genera. In addition, the ITS region was readily amplifiable for most fungi, and was consequently, adopted as the formal DNA barcode for fungi. By December 2012, about 300,000 fungal ITS sequences were available in public databases (Kõljalg *et al.,* 2013). The ITS region is therefore the most widely sequenced genetic marker in fungi and has been utilised for identification and phylogenetic analysis of fungi (Ryberg *et al.,* 2009). Despite this, the ITS sequences in the International Nucleotide Sequence Databases (INSD) represent only 1% of the hypothesised fungal population (Ryberg *et al.,* 2009).

The ITS region has been used to delineate between genus and species in different Phyla. A large-scale analysis of all available Basidiomycota sequences in the GenBank, for example (Bickford *et al.*, 2006) indicated that complete ITS region and sub-regions were robust in discriminating most Basidiomycota genera. Similar studies have also been undertaken in other fungal genera (Donnell, 1992; Kusaba & Tsuge, 1995; Skouboe *et al*., 1999; Cooke *et al.,* 2000; Nilsson *et al.,* 2008; Weir *et al.,* 2012). Taken together, these studies confirm that ITS region is a robust marker for identification and phylogenetic analyses of most fungi genera.

Ribosomal DNA (rDNA) occurs as an array of tandem repeats and is found in multiple copies in the genome of both prokaryotes and eukaryotes (Iwen *et al.,* 2002). The structure and function of rDNA has been widely studied in yeast (Fernández-Pevida *et al.,* 2015). Ribosomal DNA is composed of three ribosomal RNA (rRNA) genes namely the 18S, 5.8S and 26S genes. The 5.8S gene is flanked by two internal transcribed spacer regions (ITS1 and ITS2). The 18S-ITS1-5.8S-ITS2-26S unit is interspaced by the non-coding intergenic spacer region (IGS). The external transcribed spacer regions, ET1 and ET2 are located in the IGS region. Thus from the 5'-3' orientation, the sub-unit consist 5'-ETS1/18S/ITS1/5.8S/ITS/28S/ETS2-3' (Figure 3-1). The sub-unit is referred to as the 35S to 45S rDNA transcription unit (Iwen *et al.,* 2002).

rDNA is transcribed at high efficiency and it is estimated that pre-rRNA transcripts contribute 60% of total nuclear transcripts (Warner, 1999). The 35S to 45S rDNA transcription unit is transcribed by RNA polymerase (RNAP1) to form the 35S to 45S pre-rRNA transcription unit. The spacer regions are then enzymatically cleaved leading to formation of a primary rRNA, which undergoes RNA methylation and is assembled into an 80S nucleolar ribonucleoprotein

particle (RNP). The 80S RNP is cleaved to produce 18S, 5.8S and 28S mature rRNA. These mature rRNAs combine with proteins and 5S rRNA to form large (60S) and small (40S) ribosomal subunits, which are involved in amino acid biosynthesis and ultimately in protein synthesis (Iwen *et al.,* 2002).

The ITS and ETS regions have been shown to play a role in synthesis of ribosomes. In yeast (Musters *et al.,* 1990) deletion of ETS and ITS1 prevented accumulation of 17S rRNA and its assembly to 40S sub-units. The 60S sub-unit was not affected by deletion of ITS and ETS regions. Deletion of a small portion of the 5' end of ITS2 blocked maturation of 26S and affected the secondary structure of ITS2. The deletion, however, had no effect on maturation of 17S rRNA (Van der Sande *et al*., 1992; Good *et al.*, 1997). Overall, these studies show that spacer regions have a role to play in the synthesis of specific rRNA.

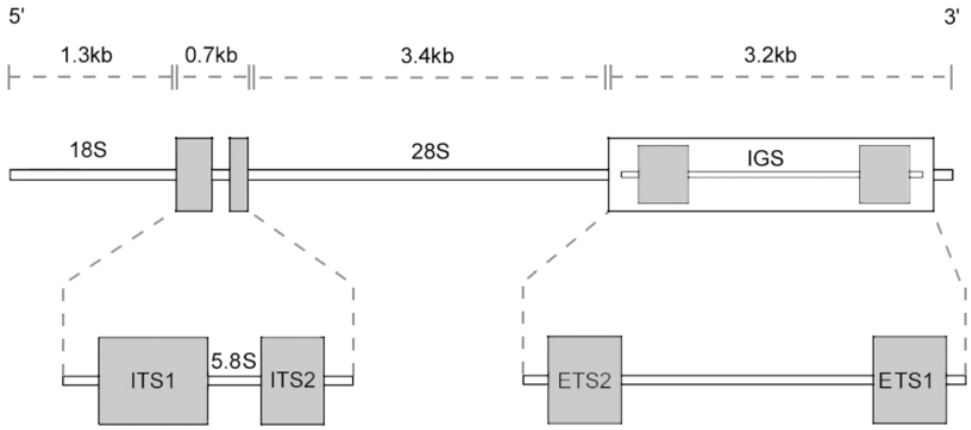


Figure 3-1. A diagrammatic representation of the rDNA repeat unit in *Saccharomyces cerevisae*. The diagram is scaled to approximate size of each region. The 18S-ITS1-5.8S-ITS2-28S units are repetitive in the fungal genome with each unit interspaced by the IGS region (Iwen *et al*., 2002).

Although the ITS region is the most widely sequenced fungal genome fragment, a number of limitations have been reported in the utilisation of the publicly available ITS sequences for fungal species identification. Only half of these sequences are annotated to the level of species (Schoch *et al.,* 2012). Moreover,

about 20% of the sequences are wrongly annotated (Bridge *et al*., 2003; Nilsson *et al.,* 2006). Compared to the saprotrophs, mycorrhizal and parasitic fungi account for most of the insufficiently annotated fungi. The genus *Alternaria, Tomentella* and *Glomus* have the highest number of insufficiently annotated ITS sequences (Ryberg *et al.*, 2009). Poor quality sequences mainly comprising of chimera sequences have also been reported. The occurrence of chimera sequences may be due to misidentification of the original material or poor isolation techniques leading to contaminated DNA (Bridge *et al.,* 2003). Due to these drawbacks, efforts were made by consortia of scientists, including taxonomists, ecologists and bioinformaticians to validate and develop reliable identification systems for fungi. These efforts culminated into the development of User-friendly Nordic Ectomycorrhizal Database (UNITE), a curated ribosomal DNA sequence database (Kõljalg *et al.,* 2005). The first version of UNITE database was released in 2003 and contained mainly sequences of ectomycorrhizal fungi from northern Europe. Subsequent developments of the database increased the scope to cover fungi from all geographical locations.

In 2013, a workshop comprising of fungal taxonomists was held in University of Tartu, Estonia with the sole purpose of annotating ITS sequence in the extended UNITE database (Kõljalg *et al.,* 2013). UNITE utilises a two-tier clustering system in which sequences are first clustered into approximately subgenus/genus level. In the second clustering, the sequences are clustered approximately to species level. The taxa generated by the second level of clustering are referred to a species hypotheses (SHs), which comprise multiple sequences. Representative sequence for each SH is computed automatically by generating consensus sequence and identifying which SH sequence best matches the consensus sequence. Alternatively, a reference sequence may be manually selected based

on expertise assessment by a taxonomist. The assessment criteria is based on type status, source material and quality of the sequence. The representative and reference sequences are taxonomically annotated and metadata including type status, voucher specimen/culture, country of origin, and substrate of collection are added. The representative and reference sequences are made available for similarity searches. Sequence similarity search can be performed using BLASTn or by phylogenetic sequence identification using galaxie (Kõljalg *et al.*, 2005).

## 3.2 *Magnaporthe oryzae* transposons and their application in population genetics

Various transposable elements have been described in the *Magnaporthe oryzae* genome (Dean *et al*., 2005). A family of Magnaporthe repeat sequences (MGR) was, for example, first identified by Hamer *et al.* (1989). The MGR sequences are distributed among all chromosomes and comprised of at least four members with different, but contiguous, sequences. The study indicated that the MGR sequences resembled other retrotransposons, for example Ty elements in *Saccharomyces cerevisiae* that encode for both a large poly $(A)^+$ RNA and a smaller poly $(A)^+$ RNA. This suggested that MGR sequences may contain RNA polymerase II promoters and encode for translatable products. Two members of the MGR sequences namely pCB583 and pCB586 (later renamed MGR583 and MGR586, respectively) were highly conserved among rice-infecting isolates compared to grass-infecting isolates of *M. oryzae*. This indicates that MGR sequences are associated with the evolution of rice-specific pathogen genotypes of the fungus. Furthermore, pCB586 sequences were sufficiently polymorphic to differentiate between different rice-infecting isolates and therefore suitable for determining genetic diversity in DNA fingerprinting population studies. A plasmid harbouring MGR Sequences, namely pCB586, has been used widely as a probe

in rice blast DNA fingerprinting studies. The MGR586 element has further been characterised by Farman *et al.* (1996), as being 1860 bp long and containing 42 bp of inverted terminal sequences. MGR586 contains the Pot3 retrotransposon and shares 23% amino acid identity with Pot2 and Fot1 elements, which themselves share 36% amino acid identity. However, the three elements have no similarity at the DNA sequence level. The study findings also indicated that pCB586 contained truncated sequences of MGR586 and was flanked by a significant amount of single-copy DNA. These findings by Farman *et al.* (1996) were critical in interpreting results of an earlier study undertaken by Hamer *et al.* (1989). In that study, Hamer and colleagues showed that MGR586 sequences were absent in grass-infecting isolates but noted faint hybridisation between the pCB586 probe and genomic DNA of the isolates. This hybridisation is now attributed to the single-copy region in the pCB586 probe. These findings affirmed that the MGR586 element is absent in grass-infecting isolates and support the hypothesis that MGR586 was introduced into *M. oryzae* during the diversification of rice-infecting isolates. Pot2 (*Pyricularia oryzae* transposon) is 1857 bp long transposable element with inverted 43 bp terminal repeats (TIRS) and is dispersed in all the chromosomes (Kachroo *et al.,* 1994). Pot2 is closely related to Fot1 transposable element found in *Fusarium oxysporum* and shares a 40% similarity in their predicted peptide sequence. Pot2 transposable element has a copy number of approximately 100 per genome in both rice and non-rice infecting isolates indicating that it is an ancestral element. The ancient origin of Pot2 element has further been affirmed by (Eto *et al.,* 2001). The study compared distribution of various transposable elements in *Magnaporthe* spp infecting various gramineous host plants. The transposable elements, Pot2, MGR586, MGR583 and Mg-SINE were widely distributed among the isolates tested.

However, MAGGY and *Grasshopper* elements show limited distribution, suggesting that they are relatively new elements that may have been acquired through horizontal transfer. The *Grasshopper* element is almost exclusively found in isolates of *M. oryzae* infecting *Eleusine* spp (Dobinson *et al.,* 1993; Kachroo *et al.,* 1994). However, in a few exceptional cases, the *Grasshopper* element has been identified in rice-infecting isolates (Mahesh *et al.,* 2016). The authors hypothesis that the presence of *Grasshopper* in rice-infecting isolates could be as a result of gene flow between rice and non-rice *Magnaporthe* populations. In that study isolates were collected from a rice-finger millet cropping system where the two crops are co-cultivated. *In vitro* mating tests between rice and finger millet isolates indicated that the two populations are capable of crossing and producing perithecia (Mahesh *et al.,* 2016). This suggests the possibility of sexual exchange of genetic material between the two populations. Although isolates from opposite mating types can successfully mate under laboratory conditions, sexual reproduction has not been observed in the field. It has been suggested that rice infecting *M. oryzae* isolates may have lost female fertility during its dispersion from Asia to other regions worldwide. Currently however, there is no direct evidence to confirm that sexual reproduction in *Magnaporthe* occurs in nature (Saleh *et al.,* 2012).

A transposon can be inserted within another transposon, as exemplified by a study by Kachroo *et al.* (1995). Mg-SINE (*Magnaporthe grisea* SINE) was identified as a 0.47 kb long insertion element within Pot2. Mg-SINE is dispersed among all the chromosomes and present in both rice and non-rice infecting isolates. The Mg-SINE element showed typical features of other SINE elements including presence of A-and B-box consensus sequences that perfectly matched the tRNA polymerase III promoter consensus sequences. The presence of the

element in both rice and non-rice infecting isolates suggests that this is an ancient element that arose before the host- specific genotypes emerged.

Among the known *Magnaporthe* transposons, MGR586, Pot2 and Maggy have been used to define the genetic structure of rice blast isolates in a population. MGR586 was first used to define the population structure of rice blast isolates in the United States (Levy *et al.,* 1991). The authors showed that the population structure of rice blast isolates in United States, comprised 8 distinct clonal lineages. A relationship was observed between the clonal lineages and the pathotype. Six of the eight lineages comprised a single pathotype, while two lineages comprised multiple pathotypes. Using a blind-test design, pathotypes of isolates collected over a 30-year period were accurately identified based on their similarity of fingerprints with reference isolates of known pathotype. However, subsequent studies by (Xia *et al.,* 1993), established a more complex relationship between the lineages and pathotypes in the United States. The authors analysed a collection of 130 isolates from two commercial fields in Arkansas and confirmed the existence of seven of the eight lineages earlier identified by Levy *et al.* (1991). However, the lineages showed heterogeneity in their virulence with multiple pathotypes occurring within a lineage. MGR586 has been used extensively to define the population of rice blast in other rice growing regions worldwide, including Columbia (Levy *et al.,* 1993), Europe (Roumen *et al.,* 1997), The Philippines (Chen *et al.,* 1995) Japan (Don *et al.,* 1999), Korea (Park *et al.,* 2003), India (Kumar *et al.,* 1999) and Africa (Takan *et al.,* 2012). Taken together, these studies support the hypothesis that the rice blast population in most rice-growing regions is comprised of distinct clonal lineages that are asexually propagated. However, in South East Asia, where there is a greater diversity of the pathogen, the *Magnaporthe* population structure is more complex and lacks such distinct

clonal lineages (Saleh *et al.,* 2014). This suggests the occurrence of sexual reproduction and recombination in the population, which corresponds with the site of origin of *M. oryzae* and thus its centre of diversity.

Pot2 DNA fingerprinting was first utilised by George *et al.* (1998) to define the population structure of rice infecting and non-rice infecting rice blast isolates from The Philippines and India. The DNA fingerprinting pattern was host-dependent, with rice infecting isolates having banding patterns distinct from the non-rice infecting. Subsequently, it has been used to define the population structure of rice blast from various rice growing regions worldwide including Americas (Xing *et al.,* 2013), Europe (Piotti *et al.,* 2005) China (Chen *et al.,* 2006). Consistent with the earlier MGR586 studies, DNA fingerprinting with Pot2 element supports the hypothesis of occurrence of distinct clonal lineages in most rice growing areas and a lack of distinct clonal lineages in regions, where there is diversity of the disease.

Traditionally, characterisation of genetic diversity of *M. oryzae* has relied on restriction fragment length polymorphism (RLFP) and Southern blotting with MGR586 sequences as the probe. For the majority of studies aimed at defining genetic diversity of a population, large number of isolates are analysed simultaneously, making it a very time-consuming activity. DNA fingerprinting with Pot2 takes advantage of the flexibility of the Polymerase Chain reaction (PCR) to generate polymorphic DNA fragments and fingerprinting patterns that define genetic diversity of isolates. Correspondence between polymorphism generated by MGR RLFP and Pot2 repetitive element based-PCR (Pot2 rep-PCR) has been reported (George *et al,* 1998). This confirms the robustness of Pot2 rep-PCR in defining the genetic structure of a rice blast population. Due to its reliability and ease of application, the Pot2 element has recently emerged as a genetic marker

of choice in most contemporary studies aimed at defining the genetic diversity of *M. oryzae* (Chen *et al.,* 2006; Xing *et al.,* 2013; Shang *et al.*, 2016).

Pot2 rep-PCR comprises of outwardly directed primers that amplify intervening sequences between any two Pot2 elements within a PCR amplifiable distance at a chromosomal locus. The amplicons are resolved by fractionating them on an agarose gel by electrophoresis. Isolates sharing a large number of bands (typically, sharing ≥70% of the bands) are considered to comprise a single lineage. The isolates within a lineage are inferred to have originated from a common ancestor.

Although rice blast disease is a major rice disease in Kenya (Kihoro *et al.,* 2013), there is limited information regarding the genetic diversity of *M. oryzae* isolates in the rice growing areas. Understanding the genetic diversity of rice infecting *M. oryzae* isolates is a critical component of an integrated and sustainable rice blast disease management strategy. In this study, we aimed to clarify the genetic diversity of *M. oryzae* isolates from the rice-growing regions in Kenya by examining the DNA fingerprinting patterns based on the distribution of the Pot2 element and by DNA sequence analysis of the ITS region of rDNA in *M. oryzae*.

### 3.3 Characteristics and occurrence of mating types in *M. oryzae*

The perfect stage of *M. oryzae* was first reported and described by Hebert (1971) and is known to be controlled by a single locus, *MAT1* (Turgeon and Yoder, 2000). The *MAT1* locus in *M. oryzae* was cloned from sexually reproducing laboratory isolates by genomic subtraction method (Kang *et al.,* 1994) and its organisation is reported to be similar to other ascomycetes (Kanamori *et al.*, 2007). The *MAT1-1* idiomorph contains a 3.5 kb open reading frame designated *MAT1-1-1* and encodes a protein with an alpha–box DNA binding motif. The

opposite mating type gene, *MAT 1-2,* contains a 2.5 kb open reading frame designated *MAT1-2-1* which encodes for a protein with an HMG-box DNA-binding motif. The study also identified novel mating type dependent ORFs named *MAT1-1-3* and *MAT1-2-2* associated with *MAT 1-1* and *MAT1-2*, respectively.

In sexually reproducing pathogens, hybridisation is an important phenomenon that leads to pathogenic variation. For sexual reproduction to occur in heterothallic species, for instance, at least one of the mating pairs must have the capacity to produce female organs (perithecia) in which the process of meiosis takes place. This ability to form female organs is often referred to as female fertility (Saleh *et al.,* 2012). *M. oryzae* is heterothallic (self-incompatible) and mating is therefore only possible between strains of opposite mating type *MAT1-1* and *MAT 1-2* with either being the female fertile parent. The ability of *M. oryzae* to produce perithecia is controlled by several loci and it has been suggested that these loci segregate independently of both pathogenicity and mating type (Kolmer & Ellingboe, 1988). In that study, the authors further observed that most virulent progeny obtained from crosses between weeping love-grass (*Eragrostis curvule*) and goose-grass (*Eleucine indica*) isolates of *M. oryzae* were female sterile. The level of fertility in field isolates of *M. oryzae* is a continuum that ranges from total sterility – the inability to mate with any other isolate – to full fertility, or the ability to mate as either male or female. In addition, the level of fertility is host specific with isolates that infect weeping love grass, goose grass and finger millet (*Eleusine coracana*) being hermaphrodites. In contrast, most rice infecting isolates are female sterile and unable to produce viable ascospores when successful crosses occur with hermaphrodite strains from other grass species (Valent *et al.,* 1991; Notteghem & Silué, 1992). It has been suggested that rice infecting *M. oryzae* isolates may have lost their female fertility during the

dispersion of the fungus from Asia to other rice growing regions worldwide (Saleh *et al.,* 2012). Sexual reproduction is furthermore a polygenic trait and occurrence of female phenotypes may have been occasioned by mutations, as exemplified in *Fusarium* spp. (Hornok *et al*., 2007). Although hybridisation of *M. oryzae* has not been observed in nature, understanding the distribution of mating types in the Kenyan rice blast fungus population provides clues to the theoretical potential of the pathogen to evolve in future and generate pathogenic variation.

## 3.4 Materials and methods

The ITS region was amplified, as described in section 2.6, from a total of 138 isolates (Table 3-1) collected from rice growing regions in Kenya.

## 3.5 Results

### 3.5.1 Phylogenetic analysis of Kenyan *M. oryzae* isolates based on the ITS sequence

Phylogenetic analysis based on the ITS sequence clustered the isolates into 5 distinct clades with clade support of >80% as shown in Figure 3-2. The majority of isolates clustered in clade 4 and clade 5. With a few exceptions, isolates from Coastal (Kwale) and Western (Homa-Bay and Ahero) Kenya clustered together in a separate clade, with those from Central Kenya (Mwea). Isolates from Mwea clustered in clade 1-4. Only 5 isolates from Ahero clustered together with Mwea isolates, whilst there were no isolates from Homa-Bay that clustered with isolates from Mwea. The clades were separated by a small number of nucleotide differences. Excluding nucleotide variations at the beginning and end of the sequence, the ITS nucleotide sequence showed only 3-4 substitutions.

Table 3-1. List of isolates used in ITS sequence analysis of Kenyan *M. oryzae* isolates

| Isolate | Variety sampled | Part of plant sampled | Location of Collection | GPS coordinates | Year Sampled |
|---|---|---|---|---|---|
| KE0001 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 38.23''/ E 37º 21' 39.68'' | 2013 |
| KE0002 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 38.23''/ E 37º 21' 39.68'' | 2013 |
| KE0003 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 38.23''/ E 37º 21' 39.68'' | 2013 |
| KE0006 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0008 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0009 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0010 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0011 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0013 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0014 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0015 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0016 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.29''/ E 37º 21' 46.35'' | 2013 |
| KE0017 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.14''/ E 37º 21' 46.25'' | 2013 |
| KE0019 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.14''/ E 37º 21' 46.25'' | 2013 |
| KE0020 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0021 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0022 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0023 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |

| | | | | | |
|---|---|---|---|---|---|
| KE0027 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 15.71''/ E 37º 22' 39.32'' | 2013 |
| KE0029 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 15.71''/ E 37º 22' 39.32'' | 2013 |
| KE0030 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 59.50''/E 37º 22' 49.36'' | 2013 |
| KE0034 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50''/E 37º 22' 44.36'' | 2013 |
| KE0035 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50''/E 37º 22' 44.36'' | 2013 |
| KE0036 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50''/E 37º 22' 44.36'' | 2013 |
| KE0037 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50''/E 37º 22' 44.36'' | 2013 |
| KE0040 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50''/E 37º 22' 44.36'' | 2013 |
| KE0200 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50''/E 37º 22' 44.36'' | 2014 |
| KE0201 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0202 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0203 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0204 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0205 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0206 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0207 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0208 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43''/E 34º56' 14.61'' | 2014 |
| KE0210 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.45''/E 34º 56' 14.80'' | 2014 |
| KE0212 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0214 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.45''/E 34º 56' 14.80'' | 2014 |
| KE0215 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.51''/E 37º 22' 42.29'' | 2014 |
| KE0219 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.45''/E 34º 56' 14.80'' | 2014 |

| | | | | | |
|---|---|---|---|---|---|
| KE0220 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.45''/E 34º 56' 14.80'' | 2014 |
| KE0221 | Basmati 370 | Neck | Western Kenya, Ahero irrigation scheme | S 0º 38' 57.51''/E 37º 22' 44.29'' | 2014 |
| KE0222 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0223 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0224 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0225 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.51''/E 37º 22' 44.29'' | 2014 |
| KE0226 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0227 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.51''/E 37º 22' 44.29'' | 2014 |
| KE0228 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 44.77''/E 34º 56' 18.46'' | 2014 |
| KE0229 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 44.77''/E 34º 56' 18.46'' | 2014 |
| KE0230 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.88''/E 37º 22' 44.50'' | 2014 |
| KE0232 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.68''/ E 37º 22' 44.47'' | 2014 |
| KE0233 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0234 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0235 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.88''/E 37º 22' 44.50'' | 2014 |
| KE0236 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.68''/ E 37º 22' 44.47'' | 2014 |
| KE0237 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.68''/ E 37º 22' 44.47'' | 2014 |
| KE0238 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.64''/ E 37º 22' 44.53'' | 2014 |
| KE0239 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0241 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85''/E 34º 56' 18.77'' | 2014 |
| KE0244 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71''/ E34º 56' 18.37'' | 2014 |
| KE0245 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71''/ E34º 56' 18.37'' | 2014 |

| | | | | | |
|---|---|---|---|---|---|
| KE0246 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE0248 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE0249 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE0251 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE0254 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE0257 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE0301 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28' 34.27"/E 34º 57' 20.05" | 2014 |
| KE0307 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0309 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0311 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0313 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0317 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0318 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0321 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.23’’/E 34º32’ 53.10’’ | 2014 |
| KE0330 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0332 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0337 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0339 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0340 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0343 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0344 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0346 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |

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|---|---|---|---|---|---|
| KE0347 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0352 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0356 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0359 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º32’ 52.52’’ | 2014 |
| KE0360 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0366 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0368 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0369 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0372 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0374 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0375 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0376 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0377 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0379 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0385 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0386 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0387 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0388 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0389 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0390 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0392 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0395 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |

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|---|---|---|---|---|---|
| KE0396 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | |
| KE0398 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0400 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0401 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0402 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0404 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0407 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0408 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0415 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0417 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33'' 2.27'/E 39º 7'' 29.77' | 2014 |
| KE0420 | Nerica 19 | Leaf | Coastal Kenya Kikoneni, Kwale | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0430 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0432 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0433 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 32' 58.21''/E 39º 7' 44.43'' | 2014 |
| KE0436- | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.94''/E 39º 7' 30.48'' | 2014 |
| KE0437 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.94''/E 39º 7' 30.48'' | 2014 |
| KE0438 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 2.33''/E 39º 7' 29.80'' | 2014 |
| KE0439 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 2.33''/E 39º 7' 29.80'' | 2014 |
| KE0445 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 2.33''/E 39º 7' 29.80'' | 2014 |
| KE0447 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 3.33''/E 39º 7' 30.80'' | 2014 |
| KE0448 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.35''/E 39º 7' 40.80'' | 2014 |
| KE0450 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.35''/E 39º 7' 40.80'' | 2014 |

| | | | | | |
|---|---|---|---|---|---|
| KE0453 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 5''/E 39º 7' 20.80'' | 2014 |
| KE0455 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 2.33''/E 39º 7' 29.80'' | 2014 |
| KE0459 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.94''/E 39º 7' 30.48'' | 2014 |
| KE0460 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0466 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 29.47''/E 39º 6' 57.89'' | 2014 |
| KE0467 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.94''/E 39º 7' 30.48'' | 2014 |
| KE0468 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 5.33''/E 39º 7' 36.80'' | 2014 |
| KE0470 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 6.33''/E 39º 7' 35.80'' | 2014 |
| KE0475 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 5.33''/E 39º 7' 35.80'' | 2014 |
| KE0478 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 29.47''/E 39º 6' 57.89'' | 2014 |

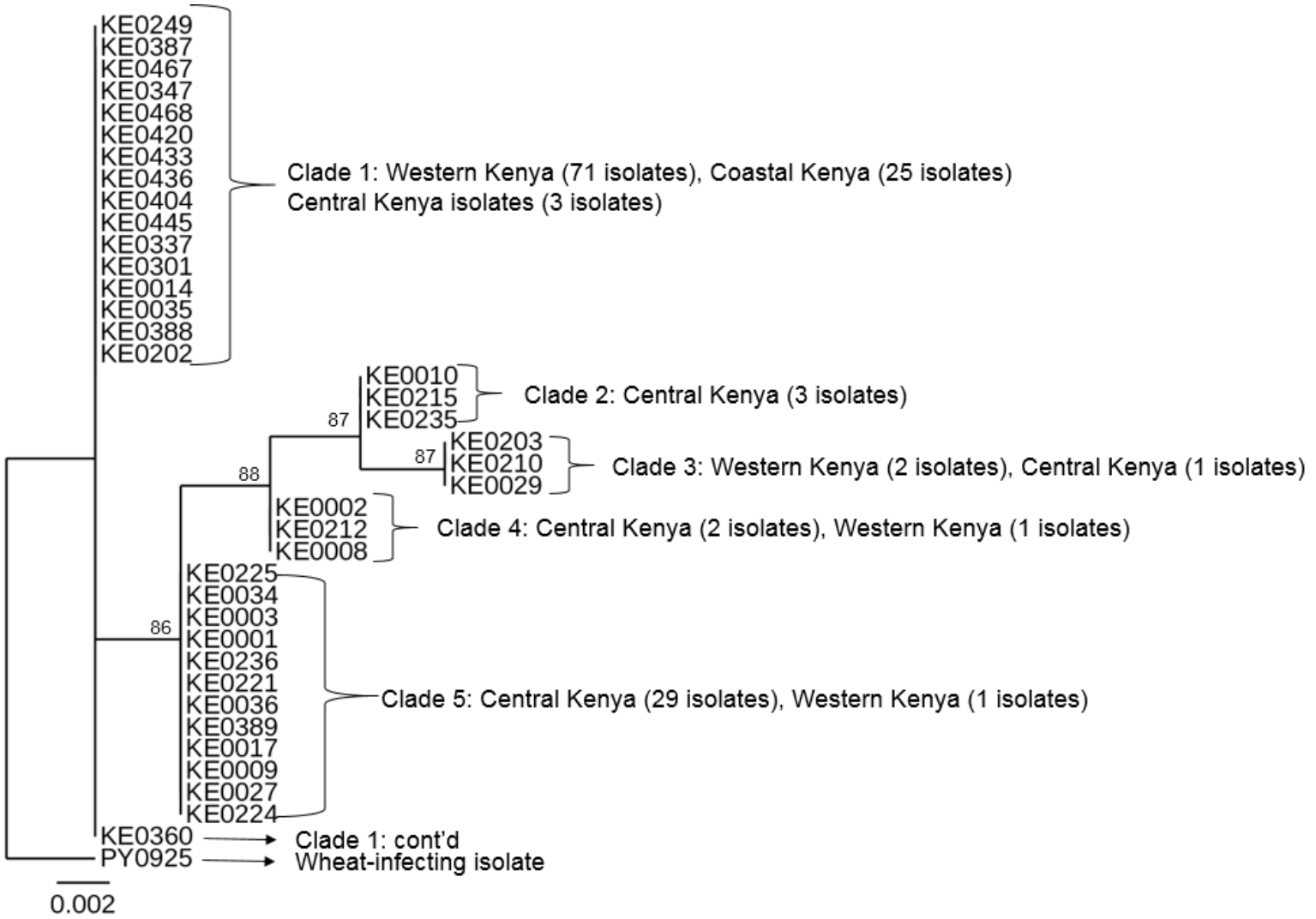



Figure 3-2. Rooted phylogenetic tree of Kenyan *M. oryzae* isolates based on the Internal Transcribed Spacer regions. Multiple sequence alignment performed with MUSCLE program. Tree constructed using maximum likelihood method on PhyML with 100 bootstraps. To make figure readable only representatives are shown. All isolates in each clade are shown in Table 3-2.

Table 3-2. List of isolates and clades generated by analysis of internal transcribed spacer region of Kenyan *M. oryzae*

| Clade/Locality | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Clade 1** | **Locality** | **Clade 2** | **Locality** | **Clade 3** | **Locality** | **Clade 4** | **Locality** | **Clade 5** | **Locality** |
| KE0235 | Central Kenya[#] | KE0203[h] | Western Kenya[++] | KE0002 | Central Kenya[#] | KE0003 | Central Kenya[#] | KE0398 | Western Kenya[+] |
| KE0215[t] | Central Kenya[#] | KE0210[j] | Western Kenya[++] | KE0008[ab] | Central Kenya[#] | KE0017 | Central Kenya[#] | KE0407 | Western Kenya[+] |
| KE0010 | Central Kenya[#] | KE0029 | Central Kenya[#] | KE0212 | Ahero | KE0009[ab] | Central Kenya[#] | KE0389 | Western Kenya[+] |
| | | | | | | KE0001[q] | Central Kenya[#] | KE0339 | Western Kenya[+] |
| | | | | | | KE0034 | Central Kenya[#] | KE0313 | Western Kenya[+] |
| | | | | | | KE0013 | Central Kenya[#] | KE0374 | Western Kenya[+] |
| | | | | | | KE0016[r] | Central Kenya[#] | KE0375 | Western Kenya[+] |
| | | | | | | KE0020[v] | Central Kenya[#] | KE0307 | Western Kenya[+] |
| | | | | | | KE0011[v] | Central Kenya[#] | KE0404 | Western Kenya[+] |
| | | | | | | KE0015 | Central Kenya[#] | KE0401 | Western Kenya[+] |
| | | | | | | KE0036 | Central Kenya[#] | KE0390 | Western Kenya[+] |
| | | | | | | KE0030 | Central Kenya[#] | KE0360 | Western Kenya[+] |
| | | | | | | KE0040 | Central Kenya[#] | KE0366 | Western Kenya[+] |

| | | | |
|---|---|---|---|
| KE0037 | Central Kenya[#] | KE0376 | Western Kenya[+] |
| KE0006[q] | Central Kenya[#] | KE0356 | Western Kenya[+] |
| KE0200 | Central Kenya[#] | KE0318 | Western Kenya[+] |
| KE0225[t] | Central Kenya[#] | KE0346 | Western Kenya[+] |
| KE0023 | Central Kenya[#] | KE0351 | Western Kenya[+] |
| KE0027 | Central Kenya[#] | KE0311 | Western Kenya[+] |
| KE0221 | Central Kenya[#] | KE0340 | Western Kenya[+] |
| KE0227 | Central Kenya[#] | KE0344 | Western Kenya[+] |
| KE0224[w] | Ahero | KE0347 | Western Kenya[+] |
| KE0021[u] | Central Kenya[#] | KE0368 | Western Kenya[+] |
| KE0220[u] | Ahero | KE0372 | Western Kenya[+] |
| KE0022[u] | Central Kenya[#] | KE0379 | Western Kenya[+] |
| KE0238[s] | Central Kenya[#] | KE0386 | Western Kenya[+] |
| KE0232[s] | Central Kenya[#] | KE0387 | Western Kenya[+] |
| KE0230[s] | Central Kenya[#] | KE0388 | Western Kenya[+] |

| | | | |
|---|---|---|---|
| KE0237[S] | Central Kenya[#] | KE0396 | Western Kenya[+] |
| KE0236[W] | Central Kenya[#] | KE0400 | Western Kenya[+] |
| | | KE0402 | Western Kenya[+] |
| | | KE0408 | Coastal Kenya[*] |
| | | KE0420 | Coastal Kenya[*] |
| | | KE0437 | Coastal Kenya[*] |
| | | KE0453 | Coastal Kenya[*] |
| | | KE0466 | Coastal Kenya[*] |
| | | KE0470 | Coastal Kenya[*] |
| | | KE0439 | Coastal Kenya[*] |
| | | KE0447 | Coastal Kenya[*] |
| | | KE0438 | Coastal Kenya[*] |
| | | KE0430 | Coastal Kenya[*] |
| | | KE0436 | Coastal Kenya[*] |
| | | KE0448 | Coastal Kenya[*] |

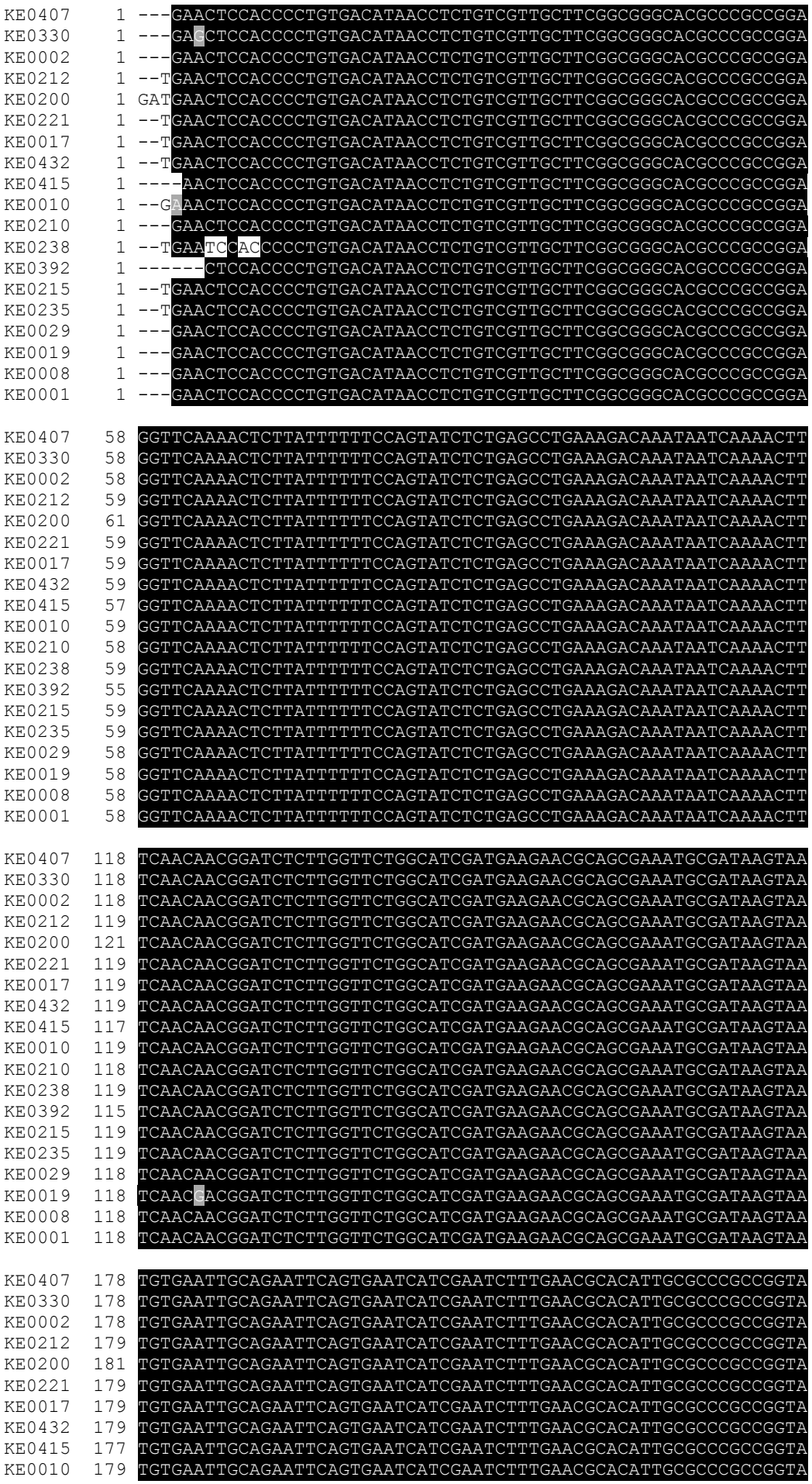
| | |
|---|---|
| KE0445 | Coastal Kenya* |
| KE0450 | Coastal Kenya* |
| KE0455 | Coastal Kenya* |
| KE0459 | Coastal Kenya* |
| KE0467 | Coastal Kenya* |
| KE0468 | Coastal Kenya* |
| KE0475 | Coastal Kenya* |
| KE0014 | Central Kenya# |
| KE0035 | Central Kenya# |
| KE0249[j] | Western Kenya++ |
| KE0369[j] | Western Kenya+ |
| KE0385[j] | Western Kenya+ |
| KE0377[j] | Western Kenya+ |
| KE0369[j] | Western Kenya+ |
| KE0222[j] | Western Kenya++ |

| | |
|---|---|
| KE0254[j] | Western Kenya[++] |
| KE0244[j] | Western Kenya[++] |
| KE0208[j] | Western Kenya[++] |
| KE0205[j] | Western Kenya[++] |
| KE0204[j] | Western Kenya[++] |
| KE0207[j] | Western Kenya[++] |
| KE0214[j] | Western Kenya[++] |
| KE0223[j] | Western Kenya[++] |
| KE0226[j] | Western Kenya[++] |
| KE0246[j] | Western Kenya[++] |
| KE0241[j] | Western Kenya[++] |
| KE0206[j] | Western Kenya[++] |
| KE0478[j] | Coastal Kenya[*] |
| KE0392[g] | Western Kenya[+] |
| KE0257[g] | Western Kenya[++] |

| | |
|---|---|
| KE0239[x] | Western Kenya[++] |
| KE0248[x] | Western Kenya[++] |
| KE0317[x] | Western Kenya[+] |
| KE0330[x] | Western Kenya[+] |
| KE0352[x] | Western Kenya[+] |
| KE0343[x] | Western Kenya[+] |
| KE0321[x] | Western Kenya[+] |
| KE0201[k] | Western Kenya[++] |
| KE0395[k] | Western Kenya[+] |
| KE0332[k] | Western Kenya[+] |
| KE0359[k] | Western Kenya[+] |
| KE0309[k] | Western Kenya[+] |
| KE0219[m] | Western Kenya[++] |
| KE0228[m] | Western Kenya[++] |
| KE0229[m] | Western Kenya[++] |

| | |
|---|---|
| KE0234[l] | Western Kenya[++] |
| KE0233[l] | Western Kenya[++] |
| KE0202[h] | Western Kenya[++] |
| KE0251[b] | Western Kenya[++] |
| KE0245[b] | Western Kenya[++] |
| KE0432[e] | Coastal Kenya[*] |
| KE0415[e] | Coastal Kenya[*] |
| KE0417[e] | Coastal Kenya[*] |
| KE0019[r] | Central Kenya[#] |
| KE0460[c] | Coastal Kenya[*] |
| KE0433[c] | Coastal Kenya[*] |

Isolates marked with the same superscript letter have identical Pot2-Pcr banding pattern as shown in Table 3-3
+ Isolates collected from Western Kenya, Homa-Bay, Maugo Irrigation scheme
++ Isolates collected from Western Kenya, Ahero irrigation scheme
# Isolates collected from Central Kenya, Mwea irrigation scheme
* Isolates collected from Coastal Kenya, Lunga Lunga and Kikoneni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KE0330 1 ---GAGCTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0002 1 ---GAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0212 1 --TGAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0200 1 GATGAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0221 1 --TGAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0017 1 --TGAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0432 1 --TGAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0415 1 ----AACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0010 1 --GAAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0210 1 ---GAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0238 1 --TGAATCCACCCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0392 1 ------CTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0215 1 --TGAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0235 1 --TGAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0029 1 ---GAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0019 1 ---GAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0008 1 ---GAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0001 1 ---GAACTCCACCCCTGTGACATAACCTCTGTCGTTGCTTCGGCGGGCACGCCCGCCGGA
KE0407 58 GGTTCAAAACTCTTATTTTTTCCAGTATCTCTGAGCCTGAAAGACAAATAATCAAAACTT
KE0330 58 GGTTCAAAACTCTTATTTTTTCCAGTATCTCTGAGCCTGAAAGACAAATAATCAAAACTT
KE0002 58 GGTTCAAAACTCTTATTTTTTCCAGTATCTCTGAGCCTGAAAGACAAATAATCAAAACTT
KE0212 59 GGTTCAAAACTCTTATTTTTTCCAGTATCTCTGAGCCTGAAAGACAAATAATCAAAACTT
KE0200 61 GGTTCAAAACTCTTATTTTTTCCAGTATCTCTGAGCCTGAAAGACAAATAATCAAAACTT
KE0221 59 GGTTCAAAACTCTTATTTTTTCCAGTATCTCTGAGCCTGAAAGACAAATAATCAAAACTT
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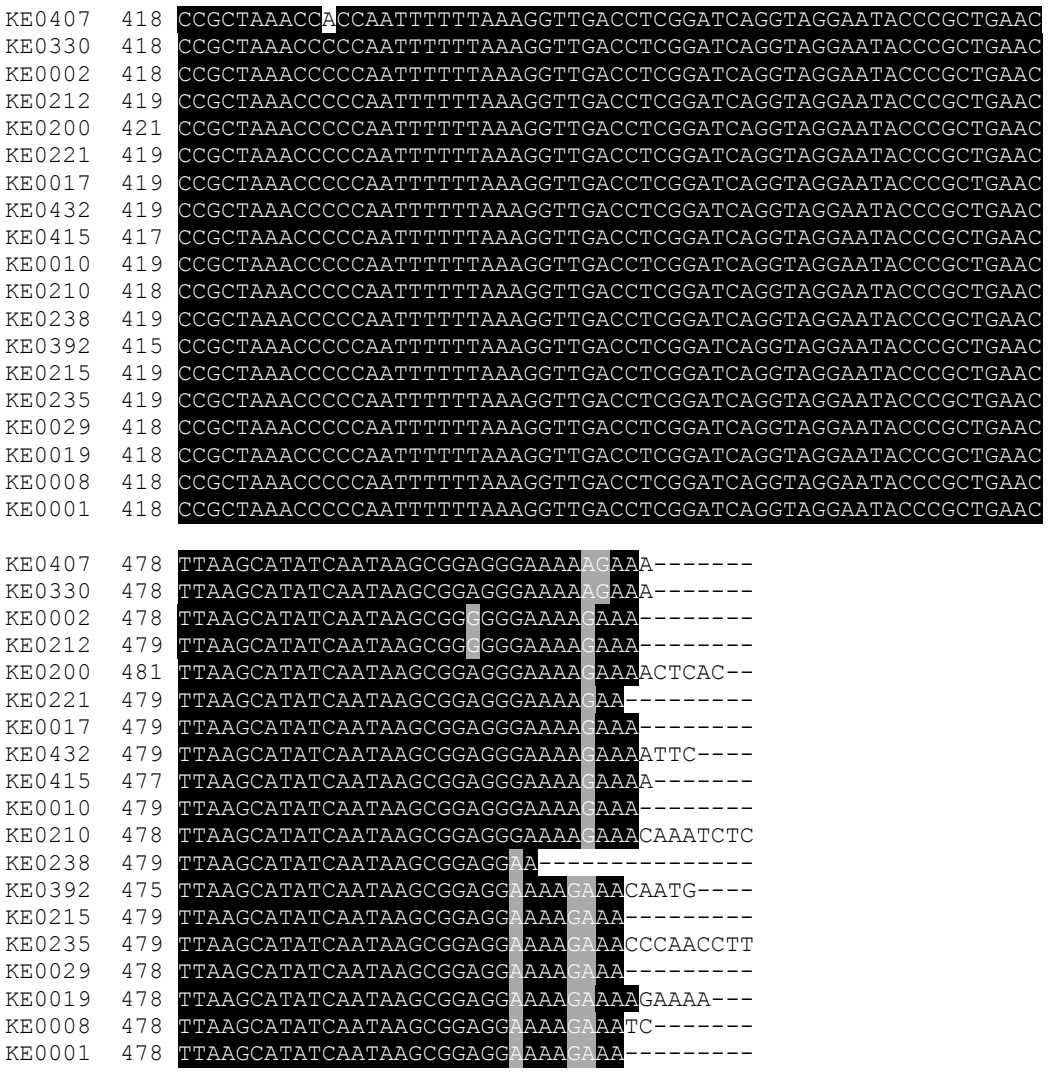


Figure 3-3. Multiple sequence alignment of ITS region of Kenyan *M. oryzae* isolates. Multiple sequence alignment performed with clustalW program. All isolates in clade 1, clade 2, clade 3 and selected isolates in clade 4 and clade 5 are presented in this figure.

### 3.5.2 Pot2 rep-PCR DNA fingerprinting of Kenyan *M. oryzae* isolates

DNA Fingerprinting was undertaken, as described in section 2.7. DNA fingerprinting patterns were generated for 153 Kenyan rice-infecting *M. oryzae* isolates and 2 isolates collected from pearl millet (*Pennisetum glaucum* L.) and Crabgrass (*Digitaria* spp.) (Table 3-3). DNA fingerprinting with the Pot2 repetitive element generated 2-12 polymorphic fragments. The fragments sizes ranged in size from 650 bp to 5 kb (Figure 3-4 – Figure 3-14). Based on approximately 80% DNA fingerprint similarity, *M. oryzae* isolates from Kenya could be classified into

5 major lineages designated KL1, KL2, KL3, KL4 and KL5 (Figure 3-15). Typical banding profiles for each lineage are shown in Figure 3-14. Lineages KL2, KL3 and KL4 comprised the largest number of isolates. The clustering of isolates was region-specific with two major groupings comprising of isolates from Central Kenya (Mwea) (lineage KL2) distinct to isolates from Coastal (Kwale) and Western region (Homa-Bay and Ahero) (lineages KL3 and KL4). Lineages KL3 and KL4 clustered as subgroups. Lineage K3 mainly comprised of isolates from Kwale (Coastal Kenya) which represented 61% of the total number of isolates within the lineage. In this lineage 34% of isolates originated from Western Kenya (Ahero and Homa-Bay). Lineage K4 mainly consisted of isolates from Ahero and Homa-Bay (Western Kenya) which represented 43% and 51% of the isolates in the lineage respectively. Most of isolates in lineage KL2 (84%) comprised of isolates from Mwea (Central Kenya). Lineage KL1 comprised of only Kwale and Homa-Bay isolates. Lineage KL5 comprised of a single isolate from Kwale (Table 3-3). Haplotypes were detected within each of the lineages. A total of 51 haplotypes were observed and percentage of haplotypes in each lineage ranged from 14-50% (Table 3-4).

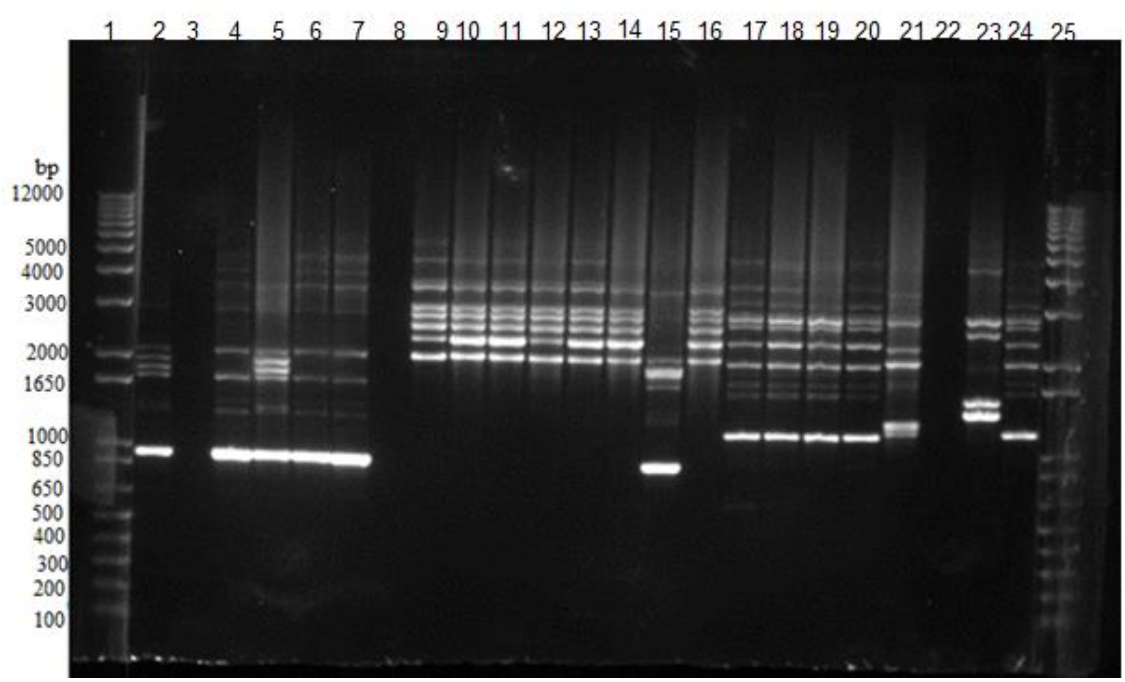


Figure 3-4. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-25; 1 Kb plus ladder, KE0001, KE0003, KE0005, KE0006, KE0008, KE0009, KE0010, KE0201, KE0204, KE0205, KE0206, KE0207, KE0208, KE0212, KE0214, KE0415, KE0416, KE0417, KE0418, KE0473, KE0488, KE0491, KE0419, 1 Kb plus ladder.

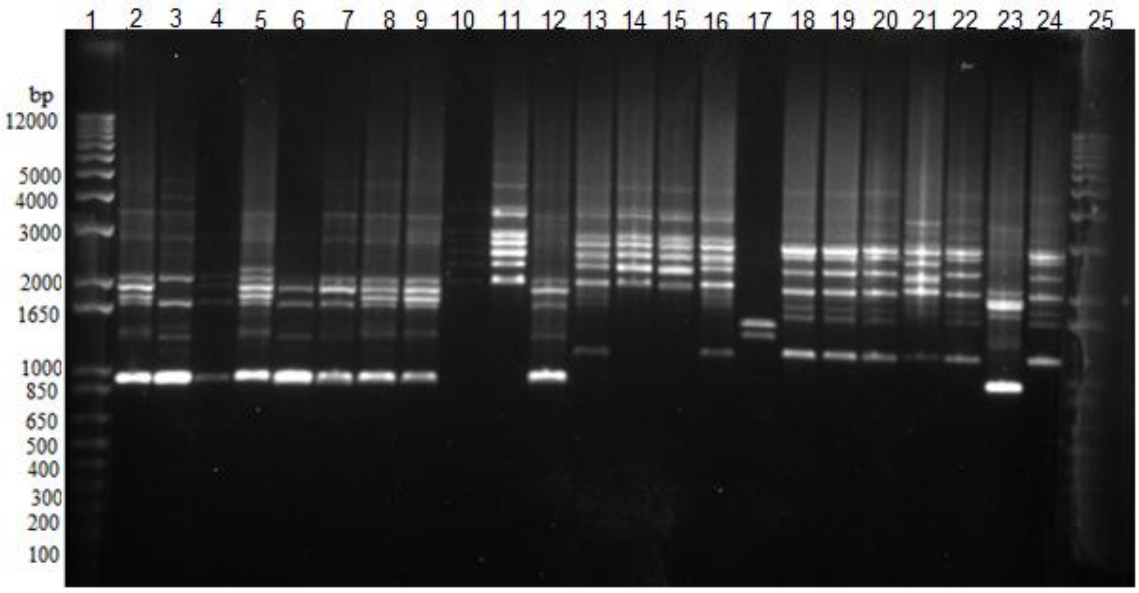


Figure 3-5. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-25; 1 Kb plus ladder, KE0011, KE0013, KE0014, KE0015, KE0017, KE0020, KE0021, KE0022, KE0217, KE0219, KE0220, KE0234, KE0228, KE0229, KE0233, KE0420, KE0423, KE0424, KE0425, KE0433, KE0442, KE0446, KE0460, 1 Kb plus ladder.

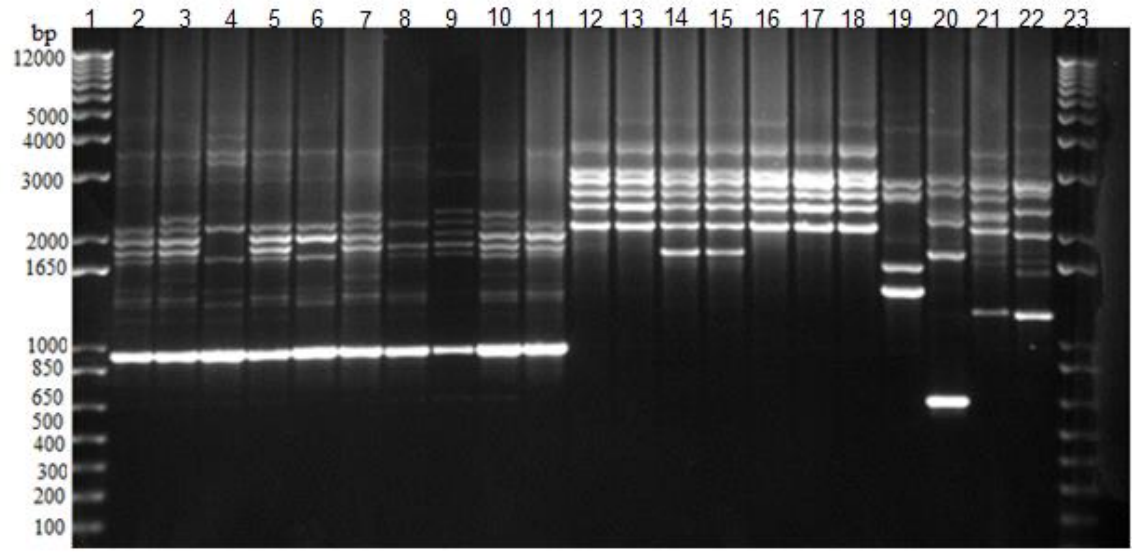


Figure 3-6. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-23; 1 Kb plus ladder, KE0021, KE0023, KE0221, KE0025, KE0027, KE0029, KE0030, KE0034, KE0035, KE0036, KE0301, KE0304, KE0306, KE0311, KE0323, KE0330, KE0336, KE0406, Guy11, KE0443, KE0446, 1 Kb Plus ladder.

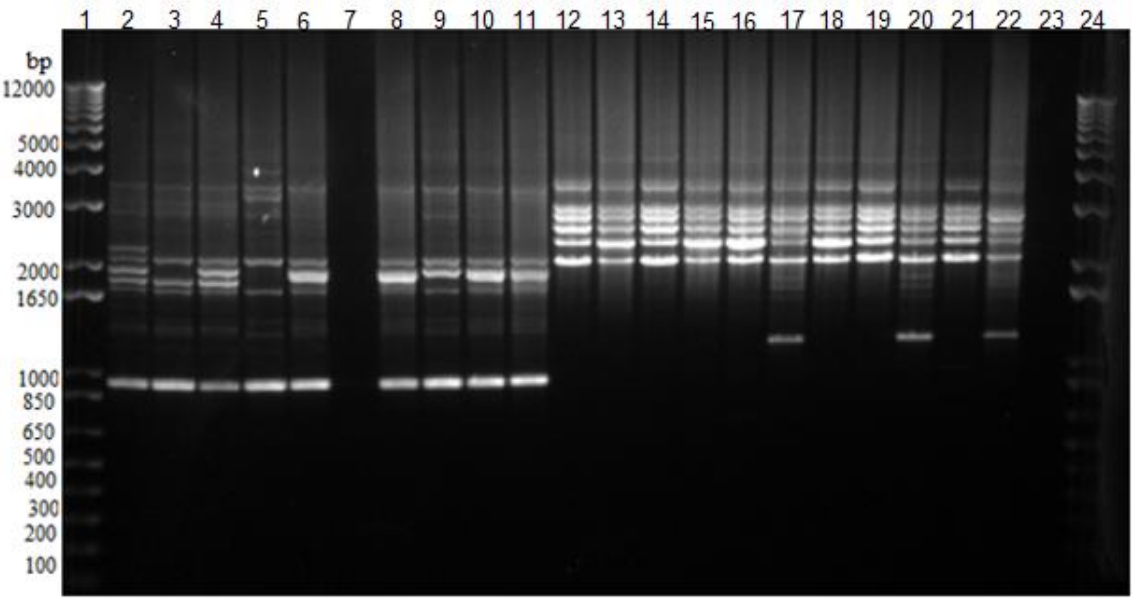


Figure 3-7. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-24; 1 Kb plus ladder, KE0037, KE0040, KE0041, KE0200, KE0225, KE0227, KE0230, KE0232, KE0238, KE0237, KE0222, KE0223, KE0226, KE0241, KE0244, KE0245, KE0246, KE0249, KE0251, KE0254, KE0255, KE0243, 1 Kb Plus ladder.

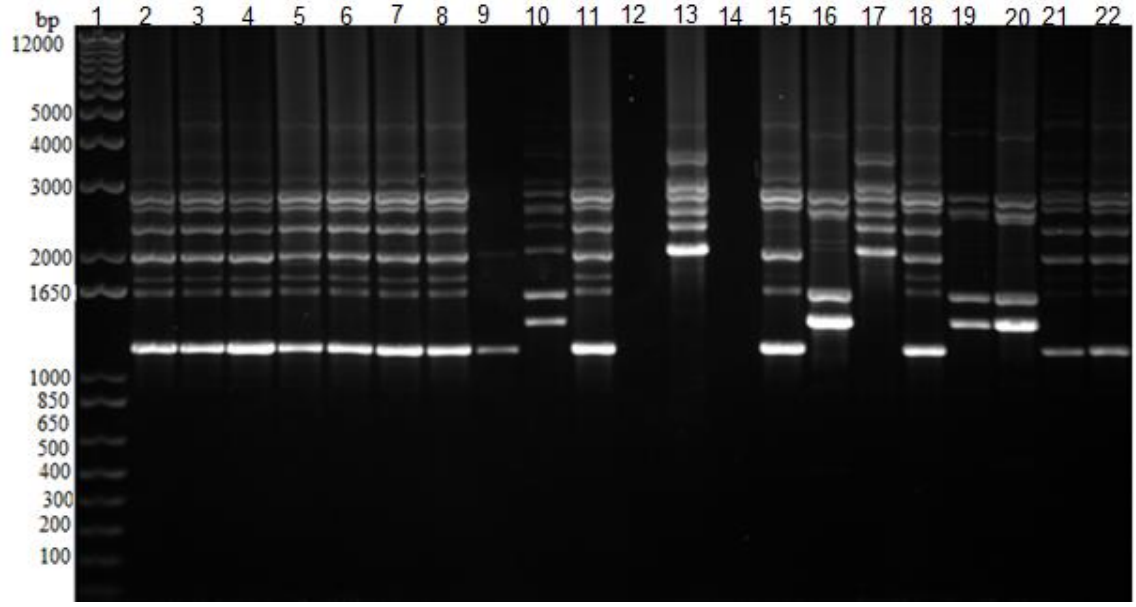


Figure 3-8. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-22; 1 Kb plus ladder, KE0490,.KE0417, KE0428, KE0453, KE0482, KE0426, KE0429, KE0432, KE0437, KE0402, KE0478, KE0470, KE0447, KE0493, KE0400, KE0466, KE0407, KE0489, KE0485, KE0416, KE0428.

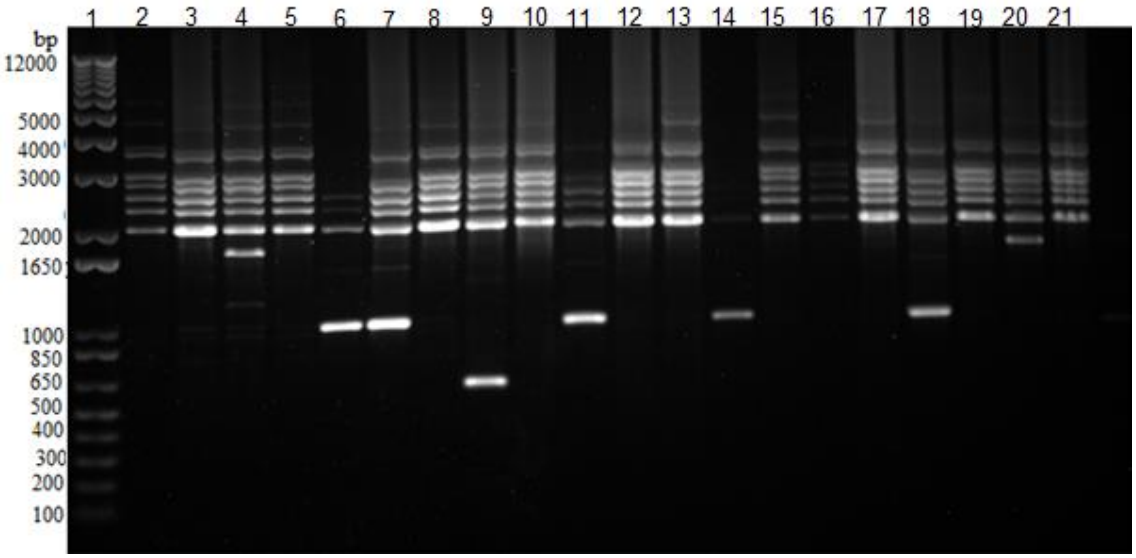


Figure 3-9. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-21; 1 Kb plus ladder, KE0359, KE0309, KE0398, KE0337, KE0379, KE0363, KE0343, KE0372, KE0317, KE0365, KE0321, KE0395, KE0374, KE0339, KE0329, KE0397, KE0361, KE0320, KE0344, KE0332.

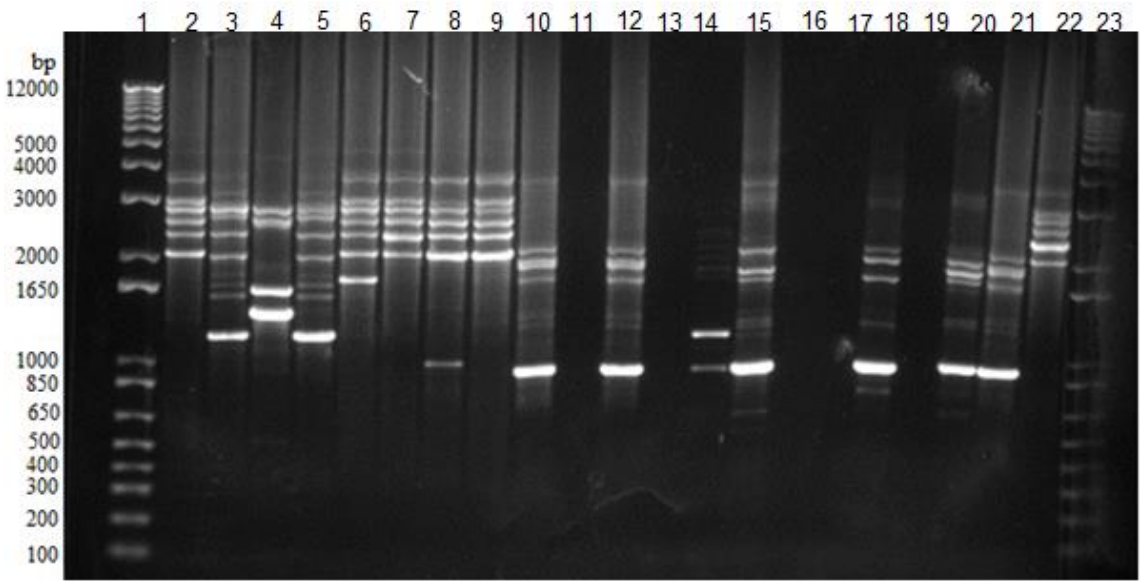


Figure 3-10. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-23; 1 Kb plus ladder, KE0385, KE0439, KE0467, KE0465, KE0313, KE0210, KE0377, KE0369, KE0236, KE0227, KE0215, KE0007, KE0002, KE0024, KE0026, KE0032, KE0019, KE0033, KE0016, KE0225, KE0202, 1 kb plus ladder.

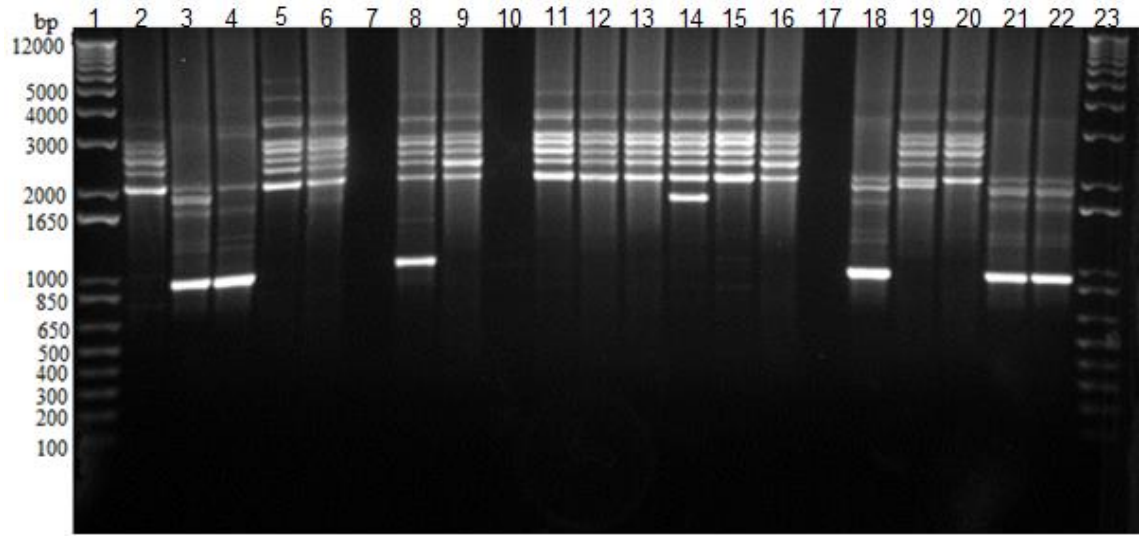


Figure 3-11. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-23; 1 Kb plus ladder, KE0203, KE0235, KE0231, KE0257, KE0389, KE0390, KE0367, KE0239, KE0386, KE0248, KE0352, KE0354, KE0393, KE0392, KE0247, KE0340, KE0252, KE0250, KE0253, KE0224, KE0236, 1 Kb plus ladder.

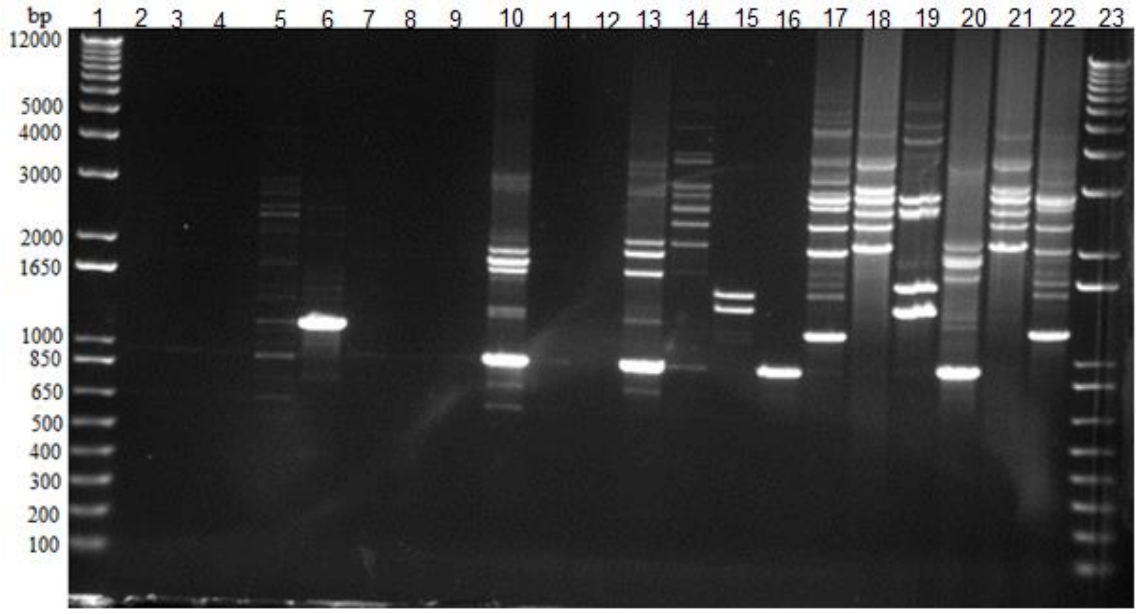


Figure 3-12. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-23; 1 Kb plus ladder, PyPA, PyKy, G17, Pm1, V107, PY36.1, PY6025, PY6017, KE0003, KE0010, KE0488, KE0014, KE0217, KE0420, KE0243, KE0432, KE0478, KE0467, KE0235, KE0369, KE0465, 1 Kb plus ladder.

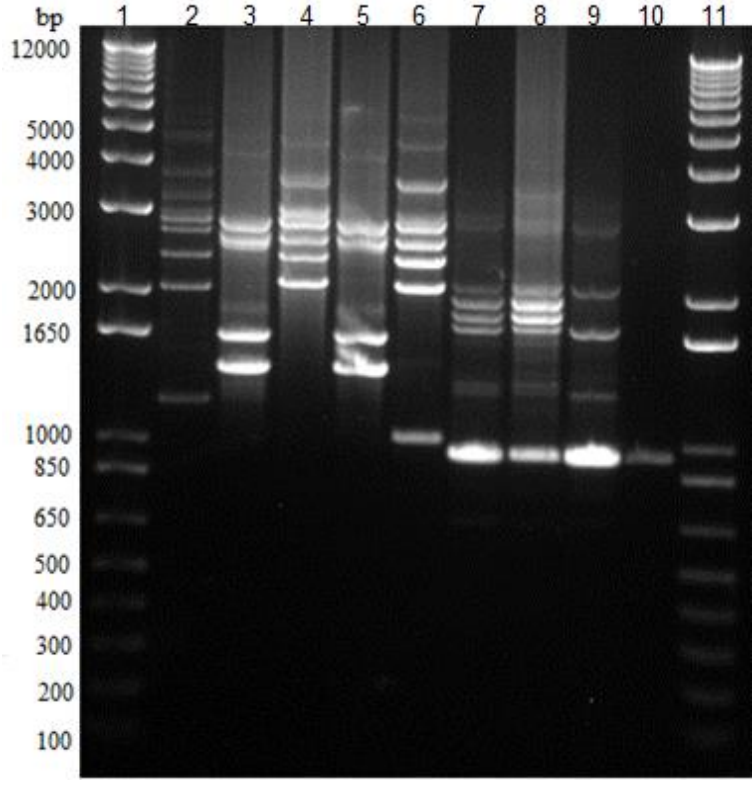


Figure 3-13. DNA fingerprinting patterns of Kenyan *M. oryzae* isolates based on Pot2 repetitive DNA element. Lanes 1-11; 1 Kb plus ladder, KE0485, KE0489, KE0400, KE0406, KE0379, KE0001, KE0006, KE0009, KE0005, 1 Kb plus ladder.

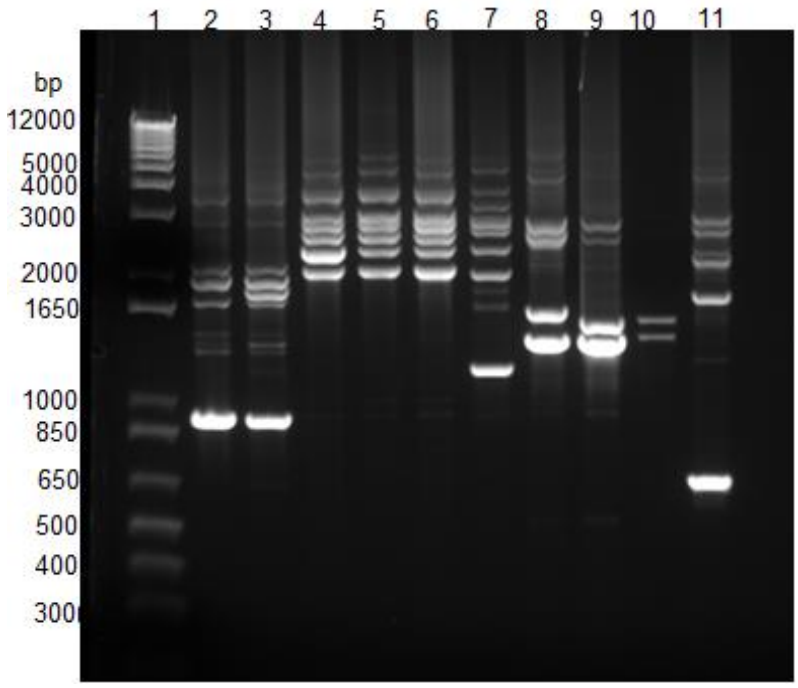


Figure 3-14. Typical banding patterns of the Kenyan *M. oryzae* lineages based on Pot2 DNA repetitive element. Lane 1: 1 Kb plus ladder; lanes 2-3 lineage KL2; lanes 4-6 lineage KL4; lane 7 lineage K3; lanes 8-9 lineage KL1; lane 10 lineage KL5, lane 11 Guy11.

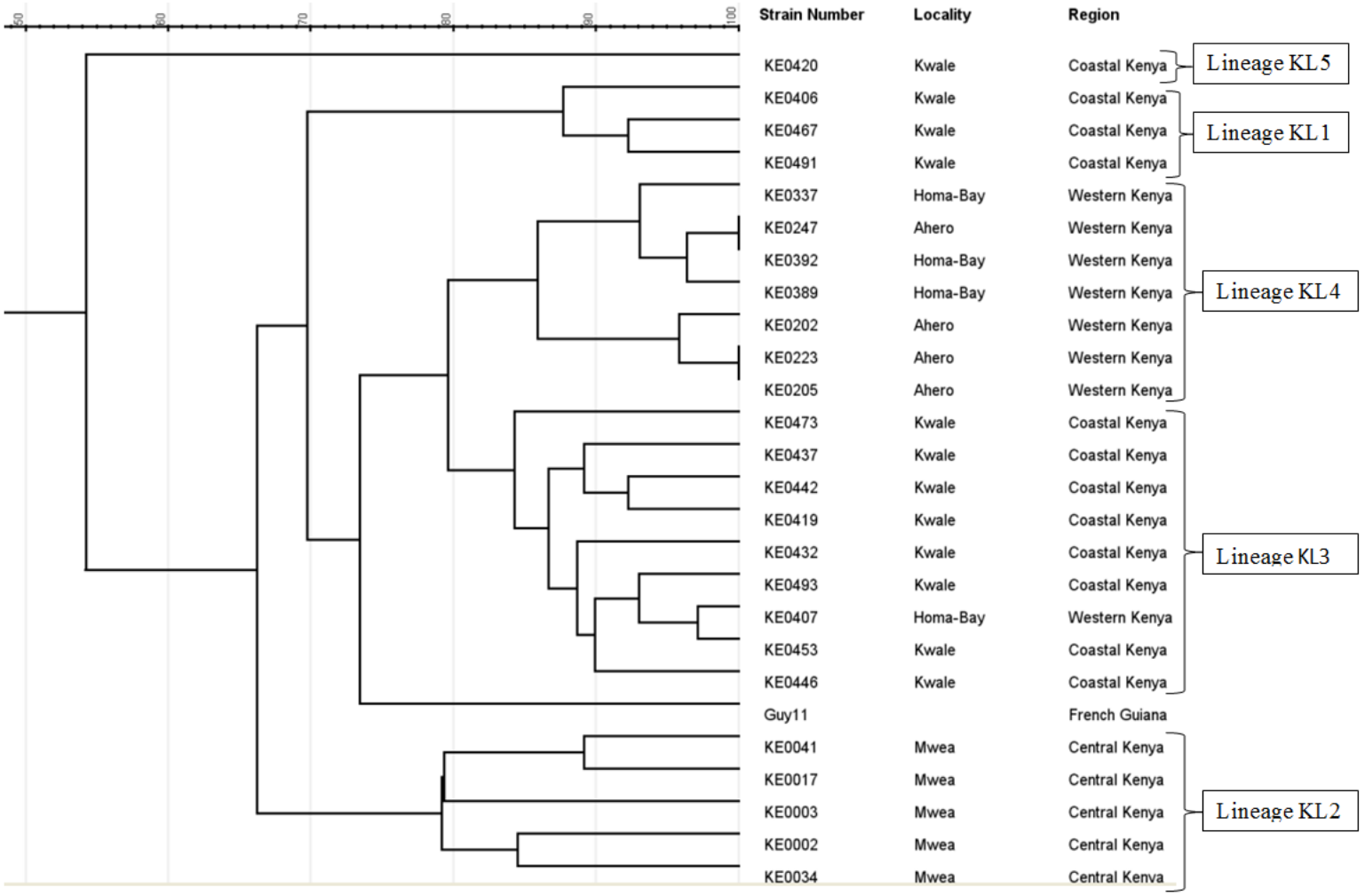


Figure 3-15. Cluster analysis of representative *M. oryzae* isolates from Kenya. Similarity matrix generated by Dice coefficient and tree constructed using Unpaired Group Arithmetic Mean (UPGMA, n=26). To make figure readable only representatives are shown. All isolates in each lineage are shown in Table 3-3.

Table 3-3. List of Kenyan *M. oryzae* lineages classified based on Pot2 DNA fingerprinting

| Lineages/Locality/ Isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **KL1** | **Locality** | **KL2** | **Locality** | **KL3** | **Locality** | **KL4** | **Locality** | **KL5** | **Locality** |
| KE0406[a] | Western Kenya[+] | KE0003 | Central Kenya[#] | KE0365 | Western Kenya[+] | KE0344 | Western Kenya[+] | KE0420 | Coastal Kenya[*] |
| KE0489[a] | Coastal Kenya[*] | KE0017 | Central Kenya[#] | KE0361 | Western Kenya[+] | KE0203[h] | Western Kenya[++] | | |
| KE0467 | Coastal Kenya[*] | KE0009[ab] | Central Kenya[#] | KE0233[l] | Western Kenya[++] | KE0202[h] | Western Kenya[++] | | |
| KE0491 | Coastal Kenya[*] | KE0002 | Central Kenya[#] | KE0234[l] | Western Kenya[++] | KE0466 | Coastal Kenya[*] | | |
| | | KE0034 | Central Kenya[#] | KE0398 | Western Kenya[+] | KE0250[f] | Western Kenya[++] | | |
| | | KE0212 | Central Kenya[#] | KE0217 | Western Kenya[++] | KE0389f | Western Kenya[+] | | |
| | | KE0013 | Western Kenya[++] | KE0363 | Western Kenya[+] | KE0400 | Western Kenya[+] | | |
| | | KE0008[ab] | Central Kenya[#] | KE0379 | Western Kenya[+] | KE0470 | Coastal Kenya[*] | | |
| | | KE0005[ab] | Central Kenya[#] | KE0493 | Coastal Kenya[*] | KE0253[x] | Western Kenya[++] | | |
| | | KE0016[r] | Central Kenya[#] | KE0367 | Western Kenya[+] | KE0247[x] | Western Kenya[++] | | |
| | | KE0019[r] | Central Kenya[#] | KE0437 | Coastal Kenya[*] | KE0336[x] | Western Kenya[+] | | |
| | | KE0024 | Central Kenya[#] | KE0473 | Coastal Kenya[*] | KE0330[x] | Western Kenya[+] | | |
| | | KE0020[v] | Central Kenya[#] | KE0460[c] | Coastal Kenya[*] | KE0304[x] | Western Kenya[+] | | |

| | | | | | |
|---|---|---|---|---|---|
| KE0011[v] | Central Kenya[#] | KE0442[c] | Coastal Kenya[*] | KE0321[x] | Western Kenya[+] |
| KE0015 | Central Kenya[#] | KE0433[c] | Coastal Kenya[*] | KE0301[x] | Western Kenya[+] |
| KE0036 | Central Kenya[#] | KE0425[c] | Coastal Kenya[*] | KE0372 | Western Kenya[+] |
| KE0030 | Central Kenya[#] | KE0424[c] | Coastal Kenya[*] | KE0339 | Western Kenya[+] |
| KE0040 | Central Kenya[#] | KE0423[c] | Coastal Kenya[*] | KE0229[m] | Western Kenya[++] |
| KE0014 | Central Kenya[#] | KE0255[b] | Western Kenya[++] | KE0228[m] | Western Kenya[++] |
| KE0037 | Central Kenya[#] | KE0251[b] | Western Kenya[++] | KE0219[m] | Western Kenya[++] |
| KE0025 | Central Kenya[#] | KE0245[b] | Western Kenya[++] | KE0323[m] | Western Kenya[+] |
| KE0200 | Central Kenya[#] | KE0407 | Western Kenya[+] | KE0313 | Western Kenya[+] |
| KE0230[s] | Central Kenya[#] | KE0453 | Coastal Kenya[*] | KE0329 | Western Kenya[+] |
| KE0238[s] | Central Kenya[#] | KE0419[e] | Coastal Kenya[*] | KE0374 | Western Kenya[+] |
| KE0232[s] | Central Kenya[#] | KE0465[e] | Coastal Kenya[*] | KE0392[g] | Western Kenya[+] |
| KE0237[s] | Central Kenya[#] | KE0418[e] | Coastal Kenya[*] | KE0257[g] | Western Kenya[++] |
| KE0224[w] | Central Kenya[#] | KE0402[d] | Western Kenya[+] | KE0241[j] | Western Kenya[++] |
| KE0252[w] | Western Kenya[++] | KE0429[e] | Coastal Kenya[*] | KE0223[j] | Western Kenya[++] |

| | | | | | |
|---|---|---|---|---|---|
| KE0236[w] | Central Kenya[#] | KE0426[e] | Coastal Kenya[*] | KE0222[j] | Western Kenya[++] |
| KE0022[u] | Central Kenya[#] | KE0482[e] | Coastal Kenya[*] | KE0254[j] | Western Kenya[++] |
| KE0220[u] | Central Kenya[#] | KE0428[e] | Coastal Kenya[*] | KE0249[j] | Western Kenya[++] |
| KE0021[u] | Central Kenya[#] | KE0417[e] | Coastal Kenya[*] | KE0246[j] | Western Kenya[++] |
| KE0035 | Central Kenya[#] | KE0416[e] | Coastal Kenya[*] | KE0244[j] | Western Kenya[++] |
| KE0029 | Central Kenya[#] | KE0415[e] | Coastal Kenya[*] | KE0226[j] | Western Kenya[++] |
| KE0023 | Central Kenya[#] | KE0432[e] | Coastal Kenya[*] | KE0369[j] | Western Kenya[+] |
| KE0041[q] | Central Kenya[#] | KE0490[e] | Coastal Kenya[*] | KE0478[j] | Coastal Kenya[*] |
| KE0001[q] | Central Kenya[#] | KE0446 | Coastal Kenya[*] | KE0210[j] | Western Kenya[++] |
| KE0006[q] | Central Kenya[#] | KE0443 | Coastal Kenya[*] | KE0385[j] | Western Kenya[+] |
| KE0235 | Central Kenya | KE0027 | Central Kenya[#] | KE0214[j] | Western Kenya[++] |
| KE0225[t] | Central Kenya[#] | KE0221 | Central Kenya[#] | KE0208[j] | Western Kenya[++] |
| KE0215[t] | Central Kenya[#] | KE0485 | Coastal Kenya[*] | KE0207[j] | Western Kenya[++] |
| KE0231 | Central Kenya[#] | KE0465 | Coastal Kenya[*] | KE0206[j] | Western Kenya[++] |
| PM1 | Central Kenya[#] | KE0439 | Coastal Kenya[*] | KE0205[j] | Western Kenya[++] |

| | | |
|---|---|---|
| V107 | KE0204[j] | Western Kenya[++] |
| | KE0377[j] | Western Kenya[+] |
| | KE0393[n] | Western Kenya[+] |
| | KE0311[n] | Western Kenya[+] |
| | KE0306[n] | Western Kenya[+] |
| | KE0343[x] | Western Kenya[+] |
| | KE0317[x] | Western Kenya[+] |
| | KE0354[x] | Western Kenya[+] |
| | KE0352[x] | Western Kenya[+] |
| | KE0248[x] | Western Kenya[++] |
| | KE0239[x] | Western Kenya[++] |
| | KE0337[k] | Western Kenya[+] |
| | KE0201[k] | Western Kenya[++] |
| | KE0359[k] | Western Kenya[+] |
| | KE0309[k] | Western Kenya[+] |

| | | |
|---|---|---|
| | KE0395[k] | Western Kenya[+] |
| | KE0397[k] | Western Kenya[+] |
| | KE0320[k] | Western Kenya[+] |
| | KE0332[k] | Western Kenya[+] |

Isolates marked with the same superscript letter have identical Pot2-Pcr banding pattern.
Isolates marked in red were presented in a dendogram as shown in Fig 3-15.
+ Isolates collected from Western Kenya, Homa-Bay, Maugo Irrigation scheme.
++ Isolates collected from Western Kenya, Ahero irrigation scheme.
# Isolates collected from Central Kenya, Mwea irrigation scheme.
* Isolates collected from Coastal Kenya, Lunga Lunga and Kikoneni.

Table 3-4. Distribution of *M. oryzae* lineages in the rice-growing regions of Kenya

| | | Haplotypes | | No. and (%) of isolates by locality | | | |
|---|---|---|---|---|---|---|---|
| **Lineage** | **No. of isolates** | **No.** | **%** | **Mwea** | **Ahero** | **Homa-Bay** | **Kwale** |
| KL1 | 4 | 2 | 50 | 0(0) | 0(0) | 1(25) | 3(75) |
| KL2 | 43 | 20 | 47 | 37(84.2) | 6(13.6) | 0(0) | 1(2.3) |
| KL3 | 42 | 19 | 45 | 2(4.8) | 7(17.1) | 7(17.1) | 25(61) |
| KL4 | 63 | 9 | 14 | 0(0) | 27(43.5) | 32(51.6) | 4(4.8) |
| KL5 | 1 | 1 | na | | | | |
| **Total** | **153** | **51** | | | | | |

### 3.5.3 Mating type distribution of Kenyan *M. oryzae* isolates

We screened 96 Kenyan *M. oryzae* isolates and two control isolates, Guy11 and TH3 of mating type *MAT1-1* and *MAT1-2,* respectively. Gene-specific primers generated PCR amplicons for *MAT1-1* and *MAT1-2*. The presence or absence of respective amplicons in the control isolates was observed, confirming the reliability of the primers in distinguishing the *M. oryzae* mating types. Both mating types *MAT1-1* and *MAT1-2* were identified in Kenyan *M. oryzae* isolates. *MAT1-1* was the predominant mating type found in all rice-growing regions of Kenya and accounts for 83% of the total number of isolates screened. The *MAT1-2* mating type was identified only in coastal Kenya (Table 3-5).

Table 3-5. Mating type distribution of Kenyan *M. oryzae* isolates

| **Isolate** | **Locality** | **Region** | ***MAT1-1*** | ***MAT1-2***** |
|---|---|---|---|---|
| KE0001 | Mwea | Central Kenya | + | |
| KE0002 | Mwea | Central Kenya | + | |
| KE0003 | Mwea | Central Kenya | + | |
| KE0005 | Mwea | Central Kenya | + | |
| KE0006 | Mwea | Central Kenya | + | |
| KE0007 | Mwea | Central Kenya | + | |
| KE0008 | Mwea | Central Kenya | + | |
| KE0009 | Mwea | Central Kenya | + | |
| KE0010 | Mwea | Central Kenya | + | |
| KE0011 | Mwea | Central Kenya | + | |
| KE0013 | Mwea | Central Kenya | + | |
| KE0014 | Mwea | Central Kenya | + | |
| KE0015 | Mwea | Central Kenya | + | |
| KE0016 | Mwea | Central Kenya | + | |
| KE0024 | Mwea | Central Kenya | + | |
| KE0032 | Mwea | Central Kenya | + | |
| KE0033 | Mwea | Central Kenya | + | |
| KE0301 | Homa-Bay | Western Kenya | + | |
| KE0304 | Homa-Bay | Western Kenya | + | |

| | | | |
|---|---|---|---|
| KE0313 | Homa-Bay | Western Kenya | + |
| KE0317 | Homa-Bay | Western Kenya | + |
| KE0309 | Homa-Bay | Western Kenya | + |
| KE0320 | Homa-Bay | Western Kenya | + |
| KE0321 | Homa-Bay | Western Kenya | + |
| KE0329 | Homa-Bay | Western Kenya | + |
| KE0332 | Homa-Bay | Western Kenya | + |
| KE0337 | Homa-Bay | Western Kenya | + |
| KE0339 | Homa-Bay | Western Kenya | + |
| KE0340 | Homa-Bay | Western Kenya | + |
| KE0343 | Homa-Bay | Western Kenya | + |
| KE0344 | Homa-Bay | Western Kenya | + |
| KE0354 | Homa-Bay | Western Kenya | + |
| KE0359 | Homa-Bay | Western Kenya | + |
| KE0361 | Homa-Bay | Western Kenya | + |
| KE0363 | Homa-Bay | Western Kenya | + |
| KE0365 | Homa-Bay | Western Kenya | + |
| KE0367 | Homa-Bay | Western Kenya | + |
| KE0369 | Homa-Bay | Western Kenya | + |
| KE0374 | Homa-Bay | Western Kenya | + |
| KE0395 | Homa-Bay | Western Kenya | + |
| KE0372 | Homa-Bay | Western Kenya | + |
| KE0377 | Homa-Bay | Western Kenya | + |
| KE0379 | Homa-Bay | Western Kenya | + |
| KE0385 | Homa-Bay | Western Kenya | + |
| KE0392 | Homa-Bay | Western Kenya | + |
| KE0393 | Homa-Bay | Western Kenya | + |
| KE0386 | Homa-Bay | Western Kenya | + |
| KE0389 | Homa-Bay | Western Kenya | + |
| KE0390 | Homa-Bay | Western Kenya | + |
| KE0397 | Homa-Bay | Western Kenya | + |
| KE0398 | Homa-Bay | Western Kenya | + |
| KE0200 | Ahero | Western Kenya | + |
| KE0201 | Ahero | Western Kenya | + |
| KE0202 | Ahero | Western Kenya | + |
| KE0203 | Ahero | Western Kenya | + |
| KE0204 | Ahero | Western Kenya | + |
| KE0205 | Ahero | Western Kenya | + |

| | | | | |
|---|---|---|---|---|
| KE0206 | Ahero | Western Kenya | + | |
| KE0207 | Ahero | Western Kenya | + | |
| KE0208 | Ahero | Western Kenya | + | |
| KE0210 | Ahero | Western Kenya | + | |
| KE0212 | Ahero | Western Kenya | + | |
| KE0214 | Ahero | Western Kenya | + | |
| KE0215 | Ahero | Western Kenya | + | |
| KE0217 | Ahero | Western Kenya | + | |
| KE0219 | Ahero | Western Kenya | + | |
| KE0419 | Kwale | Coastal Kenya | | + |
| KE0420 | Kwale | Coastal Kenya | | + |
| KE0423 | Kwale | Coastal Kenya | + | |
| KE0424 | Kwale | Coastal Kenya | | + |
| KE0425 | Kwale | Coastal Kenya | | + |
| KE0428 | Kwale | Coastal Kenya | | + |
| KE0429 | Kwale | Coastal Kenya | | + |
| KE0432 | Kwale | Coastal Kenya | | + |
| KE0433 | Kwale | Coastal Kenya | | + |
| KE0437 | Kwale | Coastal Kenya | + | |
| KE0439 | Kwale | Coastal Kenya | + | |
| KE0442 | Kwale | Coastal Kenya | | + |
| KE0443 | Kwale | Coastal Kenya | | + |
| KE0447 | Kwale | Coastal Kenya | + | |
| KE0453 | Kwale | Coastal Kenya | | + |
| KE0465 | Kwale | Coastal Kenya | | + |
| KE0466 | Kwale | Coastal Kenya | | + |
| KE0467 | Kwale | Coastal Kenya | | + |
| KE0470 | Kwale | Coastal Kenya | + | |
| KE0473 | Kwale | Coastal Kenya | | + |
| KE0478 | Kwale | Coastal Kenya | + | |
| KE0482 | Kwale | Coastal Kenya | + | |
| KE0485 | Kwale | Coastal Kenya | | + |
| KE0488 | Kwale | Coastal Kenya | | + |
| KE0489 | Kwale | Coastal Kenya | + | |
| KE0490 | Kwale | Coastal Kenya | + | |
| KE0491 | Kwale | Coastal Kenya | | + |
| KE0493 | Kwale | Coastal Kenya | + | |
| Guy 11 | | French Guiana | | + |

| | | | |
|---|---|---|---|
| TH3 | Thailand | + | |
| **Total isolates** | | **77** | **19** |
| **Mating type (%)** | | **83** | **17** |

**Mating type determined by PCR using gene specific primers with Guy 11 and TH3 (Mating type *MAT1-2* and *MAT1-1*) used as controls.

### 3.6 Discussion

ITS sequences have been used to define the phylogeny of various phytopathogenic fungi and oomycetes of different genera, including *Alternaria* (Kusaba & Tsuge, 1995), *Phytophthora* and related oomycetes (Cooke *et al.,* 2000), *Fusarium* (Donnell, 1992) and *Collectotrichum* (Weir *et al.,* 2012) among other phytopathogens. Concerted evolution leads to homogenisation of many copies of the ITS cistron and therefore it can be analysed as a single gene (Coleman, 2003). The ITS region has been used to define phylogeny of *Magnaporthe* spp. at various taxonomic levels. Takan *et al.* (2012) utilised ITS sequences to discriminate between East African blast isolates infecting *Eleusine* spp and *Digitaria* spp. In combination with other markers, Zhang and co-workers (Zhang *et al.,* 2011) have shown that the genus *Magnaporthe* and *Gaeumannocyes* are polyphyletic. For example, *M. poae,* and *M. rhizophila* were more closely related to *Gaenmannomyces* spp. than to *M. oryzae* or *M. salvinii*. Bussaban *et al.* (2005) utilised sequences of ITS region and spore morphology to delineate *Pyricularia* spp. from other closely related genera.

Analysis of ITS sequences clustered the Kenyan *M. oryzae* isolates into 5 clades. The clades were separated by only 3-4 nucleotide differences, indicating low intraspecific variability within their ITS sequences. The findings of this study are consistent with other studies that have also shown little variation in ITS sequence

within species or between very closely related species. Takan *et al.* (2012) for example reported ITS sequence dissimilarities of 0 - 0.7% and 3.5 - 3.7% within isolates infecting *Eleusine* spp. and *Digitaria* spp., respectively. Low intraspecific variability has also been reported in other fungi. The small-spored catenulate taxa of *Alternaria* is divided into six species-groups based on similarities they share in their colony and conidial characteristics (Lawrence *et al.*, 2013). Four species-groups namely *alternata, tenuissima, arbores*cens and *infectoria* were associated with *Alternaria* late blight of Pistachio in California. Isolates within the *tenuissima* and *arborescens* groups shared 100% similarity in their ITS regions. Five out of seven isolates in the alternate group had 100% similarity with two other isolates differing by only one nucleotide. In addition, *tenuissima,* and *arborescens* groups differed by a single nucleotide and clustered as a monophyletic clade. However, the *infectoria* group differed from the other groups by 70 nucleotides and clustered as a separate clade (Pryor & Michailides, 2002). In *Penicillium* spp., Skouboe *et al*.(1999) showed that several closely related taxa shared identical or nearly identical ITS sequences. No intraspecific variation was reported among the taxa except in *P. verrucosum* and *P. nalgiovense* where a single nucleotide intraspecific variation was observed.

A large-scale study aimed at analysing ITS sequences stored in the public databases reported low intraspecific variability in fungi (Nilsson *et al.,* 2008). In the study, the ITS region of 4185 species representing 973 genera was analysed with phylum Ascomycota showing intraspecific variability of 1.96%. Furthermore, Bickford *et al.* (2006) showed that the complete ITS and sub-regions were not effective in resolving 11 of the 113 genera in Basidiomycota.

In fungi, a 3% threshold value in intraspecific variability has been suggested. Nevertheless, there is no single threshold value that is appropriate across the

fungal Kingdom. For example, in *Penicillium* and *Aspergillus* a threshold value of 3% is too high, whereas in *Cantharellus* spp. a 3% threshold value is too low (Ryberg *et al.,* 2009).

In this study, DNA fingerprinting of Kenyan isolates generated 5 distinct clonal lineages. Our results are consistent with previous studies that affirm the occurrence of limited numbers of clonal lineages of *M. oryzae* in most rice-growing areas (Levy *et al.,* 1991; Chen *et al.,* 1995; Roumen *et al.,* 1997; Don *et al.,* 1999; Park *et al.,* 2003; Takan *et al.,* 2012). This is consistent with the fungus propagating clonally by means of asexual reproduction. In Kenya, rice cultivation was introduced in 1907 (MoA, 2008) and so the crop has been cultivated for a relatively short period compared to South East Asia where it is estimated that rice has been grown for over 10,000 years (Saleh *et al.,* 2014). There is limited diversity in the rice genotypes that are grown as well, and consequently limited selection pressure to drive the evolution of avirulence genes in the prevailing *M. oryzae* population.

The exception to this phenomenon is the *M. oryzae* population in South East Asia, the centre of diversity of the disease (Saleh *et al.,* 2014). It has been demonstrated that *M. oryzae* populations in India (Kumar et al., 1999) and China (Chen et al., 2006) are more complex, lacking distinct genetic lineages. These studies suggest the occurrence of sexual reproduction and more extensive recombination within *M. oryzae* populations. These regions have had very long-standing rice cultivation and growth of numerous rice genotypes over many years. This is likely to exert strong selective pressure on *M. oryzae* populations leading to wide genetic diversity.

It has been estimated that one fifth of fungi are able to reproduce asexually (Taylor *et al.,* 2015). However, the hypothesis of exclusive clonal reproduction occurring in some fungi has not been supported by population genetics and genomics testing. It is therefore now generally accepted that fungi reproduce by both recombination and clonally (Taylor *et al.,* 2015).

In the current study the Pot2 element was robust in defining the population structure of rice blast isolates from Kenya. Pot2 DNA fingerprinting patterns delineated the isolates into five lineages. The occurrence of haplotypes within the lineages suggests the possible evolution of new lineages. The lineages were region-specific with isolates from Western and Coastal Kenya clustering as sister clades that were distinct from Central Kenya isolates. This indicates that the Western and Coastal Kenya isolates of *M. oryzae* are more genetically related, compared to those from Central Kenya. This observation was supported by both DNA fingerprinting and by sequence analysis of the ITS region. Although low intraspecific variability in ITS sequences was observed, the marker sufficiently clarified the genetic diversity of Kenyan *M. oryzae* isolates.

Our study indicates that isolates from Coastal and Western Kenya clustered together in a separate clade from isolates from Central Kenya. These rice-growing regions are geographically far apart. Coastal and Western Kenya are separated by a distance of 1,000 Km, and Central Kenya is 500 Km away from either of the other regions. The occurrence of some lineages, for example KL3, in multiple locations may be explained by exchange of contaminated seed. It is estimated that 15% of rice farmers in Kenya obtain planting seed from informal systems (Kiambi & Mugo, 2016). Informal seed systems include the use of seed obtained on-farm or obtained through informal distribution systems, such as exchange between farmers, community sharing systems and local markets

(Vernooy, 2016). These informal systems may have resulted in a wider dispersal of *M. oryzae* propagules. Inter-continental dispersal of *M. oryzae* propagules has previously been reported (Tharreau *et al.*, 2009).

The genetic diversity and relationships observed in the current study may be explained by the rice cropping systems in Kenya. Rice production in central Kenya is predominated by limited number of varieties with Basmati 370 and Basmati 217 varieties, accounting for over 80% of rice produced (Kihoro *et al.,* 2013). Moreover, these varieties are susceptible to rice blast disease. Therefore the selection pressure on *M. oryzae* isolates in this region is predominantly exerted by rice blast resistance genes in Basmati type varieties. In contrast, in the Coastal and Western Kenya cropping systems the varieties used are relatively more diverse. For example, in Western Kenya the commonly cultivated varieties are IR2793-80-1, ITA 310, BW 196, Basmati 370 and Duarado precoce (Cheserek *et al.,* 2012*;* Kimani *et al.,* 2011). In addition, NERICA varieties namely NERICA 1, NERICA 4, NERICA 10 and 11 have recently been introduced (Atera *et al.,* 2011). In coastal Kenya a wide variety of locally adapted landraces are currently cultivated. Varieties and landraces grown in Western and Coastal regions may therefore be harbouring a closely related spectrum of rice blast resistance genes and therefore may exert similar selection pressure on the *M. oryzae* population.

In this study a large number of isolates were grouped into only 5 distinct lineages. Isolates sharing approximately 80% similarity in their DNA fingerprints were regarded as belonging to a single lineage and were inferred to have originated from the same ancestor. Previously, studies have shown that isolates within a clonal lineage have very closely related virulence spectrum. For this reason, it

has been suggested that a lineage should be considered as the basic unit of studying the population structure of rice blast (Levy *et al*., 1991).

Our study shows that both mating types, *MAT1-1* and *MAT1-2*, occur in Kenya with *MAT1-1* occurring at a higher frequency. In Coastal Kenya, both mating types occur together in almost equal frequency. Occurrence of both mating types in the same region has previously been reported (Onaga *et al.,* 2015; Takan *et al.,* 2012), but it is not clear why both mating types occurred only in Coastal Kenya. It may be that isolates from coastal Kenya originated from the neighbouring rice growing regions of Tanzania, as well as other parts of Kenya, providing a mixed founder population. Isolates from Coastal Kenya were collected from the border region between Kenya and Tanzania, where frequent cross border movement of people and plant material, for example, is a common occurrence. There is, however, no information available on the mating type distribution of *M. oryzae* in this region of Tanzania. Future analysis of the population structure of isolates from border regions within Tanzania will enhance our understanding on the mating type distribution observed in this study.

Although *MAT1-1* and *MAT1-2* mating types were detected in the same geographical region, sexual reproduction does not appear to be a factor that define the population structure of *M. oryzae* in Kenya. DNA fingerprint results indicated occurrence of distinct clonal lineages, consistent with an asexual mode of reproduction. Although perithecia have not been observed in nature, sexual reproduction is thought to occur in limited regions in the South East Asia (Saleh, *et al.,* 2012; Kumar *et al.,* 1999). Similarly, in finger millet (*Eleusine indica*)-rice cropping systems, the *grasshopper* element that is exclusively found in millet infecting *M. oryzae* isolates, was detected in rice infecting isolates. It was suggested that hybridisation may have occurred resulting in transfer of the

*grasshopper* element to rice infecting isolates (Mahesh *et al.*, 2016). A study by (Takan *et al.,* 2012) identified the occurrence of *MAT1-1* and *MAT1-2* millet infecting isolates in East Africa and further points out that sexual reproduction may occur in the population. Presently, millet-rice cropping system is not common in Kenya but may be adopted by farmers in future. There are sustained efforts by various agencies in Kenya to promote cultivation of drought tolerant crops, such as millet among smallholder farmers (ICRISAT, 2017). Rice and millet share similar agro-ecological zones and therefore are likely to be co-cultivated. The role of millet-rice cropping system in defining the future population of *M. oryzae* in Kenya therefore needs to be monitored.

To the best of our knowledge, this is the first study that has characterised the genetic diversity of *M. oryzae* isolates from all rice-growing regions in Kenya by DNA fingerprinting and ITS sequence analysis. The study has established a baseline of information on genetic diversity of Kenyan rice infecting *M. oryzae* isolates and provides a basis for future studies designed to assess evolution of the rice blast pathogen in Kenya. This information is essential for developing pathogen surveillance systems and sustainable rice blast disease management.

# Chapter 4: Virulence diversity of Kenyan *Magnaporthe oryzae* isolates and response of rice varieties to *M. oryzae* isolates

## 4 Introduction

### 4.1 Development of rice blast differential varieties

It has been established that *M. oryzae* shows variability in cultivar-specific virulence and this has been highlighted as the cause of frequent breakdown in rice blast resistance (Ou, 1980). Single dominant rice blast resistance genes often maintain resistance for only a few growing seasons, due to the emergence of new virulent races of the fungus. The interaction between *M. oryzae* and the host therefore conforms to the longstanding gene-for-gene theory, which states that for every resistance gene (R) in the host there is a corresponding avirulence gene (*AVR*) in the pathogen, as formulated by Flor (1971) and described for *M. oryzae* by Silué *et al.* (1992). The *AVR* genes encode for proteins that are recognized by genotypes of the host plant harbouring a cognate resistance gene. This leads to effector-triggered immunity, often accompanied by a hypersensitive reaction in which the invaded cells die, thereby limiting spread of the pathogen to other cells.

The interaction between pathogen and crop varieties may result in physiological specialisation in which different isolates show variability in the crop varieties that they are able to infect. A race (pathotype) is a group of isolates with an identical virulence spectrum on varieties of a given crop (Vale *et al.,* 2001). Races are determined by qualitative symptom development on a set of defined differential lines when isolates are applied to the host plant. The qualitative responses are classified as susceptible, resistant, moderately resistant or moderately susceptible. The potential number of races that can then be determined is

dependent on the number of differentials used, and is defined by the function $2^n$ where n is the number of differentials (Mew *et al.,* 2106).

Occurrence of *M. oryzae* physiological races was first reported in Japan in 1922 (Mew *et al.,* 2016). The first set of differential varieties comprised 12 varieties belonging to the following groups: T Group consisting of Indica varieties Te-tep, Tadukan, Usen; C group consisting of Chinese varieties Chokoto, Yakeiko, Kanto 51 and N group consisting of other Japanese varieties Shikari-shiroke, Homare-nishiki, Ginga, Norin 22, Aichi-asahi, and Norin 20. Based on this differential set, *M. oryzae* isolates were classified as N, C and T groups. N races were virulent to only N varieties; C races were virulent to C varieties and not T varieties whereas,. T races were virulent to T varieties irrespective of their virulence on C and N (Yamada *et al.,* 1972).

Until the 1960s, rice blast differential sets varied from country to country, making it very difficult to compare results. Efforts were therefore put in place to develop an international rice blast differential set of cultivars to enable comparison of results across different countries. The first international differential set was developed by Atkin and colleagues (Mew *et al.,* 2016). This comprised 8 lines *viz.* Raminad Str. 3, Zenith, NP-125, Usen, Dular, Kanto 51, Sha-tiao-tiao(s), and Caloro. The set of rice blast resistance genes harboured by the differential set varieties was, however, not known. Notwithstanding this limitation, the differential set was used to characterise *M. oryzae* from different countries including India (Padmanabhan *et al.,* 1970), Philippines (Bonman *et al.,* 1987), USA (Latterell, *et al.,* 1986), sub-Saharan Africa (Chipili, 2000). Subsequent analysis of the differential set indicated that it contained no more than four major resistance genes (R) and therefore had only a limited ability to characterise *M. oryzae* isolates. A new set of differential lines with known resistance genes was therefore

developed in Japan (Yamada *et al.,* 1972) and comprised of the following lines Shin 2 (*Pik-s*), Aichi-asahi (*Pi-a*), Ishikari-shiroke (*Pil*), Kanto 51 (*Pik*), Tsuyuake (*Pik-m*), Fukunishiki (*Piz*), Yashiromochi (*Pita*), Pi No. 4 (*Pita2*), and Toride 1 (*Piz-t*). Similarly, Kiyosawa (1984) developed another set of differentials comprising 13 R genes including Aichi-asahi (*Pi-a*), BL1 (*Pib*), Fujisaka 5 (*Pil*), Kusabue (*Pik*) ,Tsuyuake (*Pik-m*), K60 (*Pik-p*), K3 (*Pik-h*), Shin 2 (*Pik-s*), K59 (*Pit*), Yashiromochi (*Pita*) Pi No. 4 (*Pita2*), Fukunishiki (*Piz*) and Toride 1 (*Piz-t*). These differential lines were superior to the earlier differential set because they contained a greater spectrum of known R genes. The differential set has been used widely to characterise rice blast isolates (Mew *et al.,* 2016). However, the spectrum of resistance genes in the differential sets was still insufficient to characterise all rice blast isolates, and especially those from the tropics, which represent the most diverse collections. In addition, these lines harboured other unknown resistance genes in their genetic background, making it difficult to interpret results (Kobayashi *et al.,* 2007). Researchers have therefore utilised locally grown commercial varieties to supplement the differential lines. In some instances, the local varieties were able distinguish between isolates that had a similar virulence pattern on differential sets (Bonman *et al.,* 1986).

To address this insufficiency in the differential set, a joint project between the International Rice Research Institute (IRRI) and Japan International Research Centre for Agricultural Science (JIRCAS) was initiated and these efforts culminated in the development of a set of monogenic differential lines (Tsunematsu *et al.,* 2000). The monogenic lines (MLs) contained 24 major rice blast resistance genes in the genetic background of Lijiangxntuanheigu (LTH) and were developed using a backcrossing breeding method whereby a single major resistance gene was introgressed into LTH from a donor variety. LTH is a

*japonica* variety of rice from Yunnan Province of China that is highly susceptible to rice blast and in which no major resistance gene had been identified. The monogenic lines are currently the most comprehensive rice blast differential available and comprise the following R genes: *Pia, Pib, Pik, Pik-h, Pik-m, Pik-p, Pik-s, Pish, Pit, Pita, Pita-2, Piz, Piz-5, Piz-t, Pi1, PiI, Pi3, Pi5 (t), Pi7 (t), Pi9, Pi11 (t), Pi12 (t), Pi19 (t),* and *Pi20* (Table 4-1). The monogenic differential set has been used to clarify the virulence spectrum of *M. oryzae* from different rice growing regions including Asia (Li *et al.,* 2016; Tanaka *et al.,* 2016; Li *et al.,* 2016; Tsunematsu *et al.,* 2000), sub-Sarahan Africa (SSA) (Mutiga *et al.,* 2017; Nyongesa *et al.,* 2016; Akator *et al.,* 2014; Odjo *et al.*, 2014) and USA (Wang *et al.,* 2015) .

Recently Kobayashi *et al.* (2007) developed 20 near-isogenic lines under LTH, Co39 and US-2 backgrounds and contained 11 R genes including *Pib, Piz-5, Pi9, Pi3, Pia, Pik-s, Pik, Pik-h, Pi7 (*t*), Pita* and *Pita2*. Unlike the monogenic lines that showed wide variations in their agronomical characteristics (Fukuta *et al.,* 2004), the isogenic lines had similar agronomic characteristics with the background variety (Kobayashi *et al.,* 2007). In addition, the indica background in Co39 and US2 enable the lines to thrive better under tropical conditions.

## 4.2 Pathogenic variability in *M. oryzae*

Authors are in agreement that *M. oryzae* evolves rapidly and exhibits high variability in their pathogenicity (Ou, 1980, Latterell, *et al.,* 1986). However, there is no consensus on the extent of the variability. Some authors have reported a high level of pathogenic variability involving monoconidial isolates. Occurrence of pathogenic variability in rice blast isolates has been reviewed by Ou (1980b). The author reported that occurrence of pathogenic variability among monoconidial spores was a common phenomenon observed by several researchers. According

to the author, the frequent presence of multiple lesion types (susceptible, intermediate and resistant) in close proximity on leaves inoculated with single spore conidia and the great differences in lesion numbers among cultivars inoculated with the same spore suspension, is an indication of genetic and virulence variability among monoconidia isolated from such lesions. In his study, Chipili (2000) reported pathogenic variability among 10 monoconidial isolates despite their identical DNA fingerprinting patterns. Other authors found no evidence to support the hypothesis of extreme pathogenic variability in *M. oryzae* isolates (Latterell, *et al.,* 1986; Bonman *et al.,* 1987). Furthermore, *M. oryzae* has been found to be stable after long periods of storage and successive sub-culturing. Lattterell *et al.* (1986), for example, reported no changes in virulence of 50 races of *M. oryzae* under long preservation periods of 30 years. Park *et al.* (2010) also reported no apparent changes in the virulence, phenotype and DNA fingerprinting patterns generated by MGR586, MAGGY, Pot2, LINE and MG-SINE for at least up to 10 generations of sub-culturing on culture media and host plant.

Although the discrepancies in the extent of pathogenic variability may be due to differences in environmental conditions and disease assessment, a recent study has reported clonal variation in effector gene expression (Farman *et al.,* 2016). In their study, the authors indicate that differences in expression of Avr effectors among monoclonal individuals exist and propose that the response of rice to *M. oryzae* is rarely binary (resistant versus susceptible), but instead comprises an aggregate of separate responses that depend on the total concentration of Avr effectors produced by each individual. Expression of different sets of effectors among monoclonal individuals is therefore considered a bet-hedging mechanism to evade recognition by the host.

Table 4-1. List of rice blast differential monogenic lines

| **Monogenic Line[1]** | **R-gene** | **Donor line** | **Generation** |
|---|---|---|---|
| IRBL 11-ZH | *Pi11*(t) | Zhaiyeqing | $BC_2F_8$ |
| IRBL 12-M | *Pi12*(t) | RIL10 | $BC_2F_8$ |
| IRBL 19-A | *Pi19* | Aichi Asahi | $BC_1F_{10}$ |
| IRBL 1-CL | *Pi1* | C101LAC | $BC_3F_8$ |
| IRBL 20-IR 24 | *Pi20*(t) | ARL24 | $BC_1F_6$ |
| IRBL 3-CP4 | *Pi3* | C104PKT | $BC_2F_8$ |
| IRBL 5-M | *Pi5*(t) | RIL249 | $BC_3F_8$ |
| IRBL 7-M | *Pi7*(t) | RIL29 | $BC_3F_8$ |
| IRBL 9-W | *Pi9* | WHD-1S-75-1-127 | $BC_3F_8$ |
| IRBLA-A | *Pia* | Aichi Asahi | $BC_1F_{10}$ |
| IRBLB-B | *Pib* | BL1 | $BC_1F_8$ |
| IRBLI-F5 | *Pii* | Fujisaka 5 | $BC_1F_{10}$ |
| IRBLKH-K3 | *Pik-h* | K3 | $BC_1F_8$ |
| IRBLK-KA | *Pik* | Kanto51 | $BC_1F_9$ |
| IRBLKM TS | *Pik-m* | Tsuyuake` | $BC_1F6$ |
| IRBLKP-K60 | *Pik-p* | K60 | $BC_1F_8$ |
| IRBLKS-F5 | *Pik-s* | Fujisaka5 | $BC_1F_{10}$ |
| IRBLKS-S | *Pik-s* | Shin2 | $BC1F_{10}$ |
| IRBLSH-S | *Pish* | Shin2 | $BC_1F_{10}$ |
| IRBLTA 2-Pi | *Pita2* | Pi No. 4 | $BC_1F_5$ |
| IRBLTA 2-RE | *Pita2* | Reiho | $BC1F_6$ |
| IRBLTA CP 1 | *Pita* | C101PKT | $BC_5F_6$ |
| IRBLTA CT2 | *Pita* | C105TTP2L9 | $BC_3F_8$ |
| IRBLT-K59 | *Pit* | K59 | $BC_2F_8$ |
| IRBLZ 5-CA(R) | *Piz-5* | C101A51 | $BC_3F_8$ |
| IRBLZ-FU | *Piz* | Fukunishiki | $BC_1F_{10}$ |
| IRBLZT-T | *Piz-t* | Toride 1 | $BC_1F_{10}$ |

[1] Each monogenic line was developed by introgressing a single R gene in rice blast susceptible line LTH (Japonica variety) using the back crossing breeding strategy.

### 4.3 Rice varieties grown in Kenya

Common varieties grown in Kenya include Basmati 370, BW 196, and Dourado precoce, ITA 310, IR 2793-80-1, NERICA 1 NERICA 4, NERICA 10 and NERICA 11. Characteristics of these varieties are shown in Table 4-2. NERICA varieties were bred by crossing two species of rice, *Oryza glaberrima* steud. and *Oryza sativa* L. to produce progeny of interspecific siblings that contain the best traits of each variety. The objective of breeding NERICA varieties was to combine the desirable trait of high yields found in *O. sativa* and the resistance to abiotic and biotic stress found in *O. glabberima*. The development of NERICA varieties was a significant breakthrough in rice breeding because it marked the first successful attempt to overcome incompatibility between *O. sativa* and *O. glabberima*. To overcome sterility, the breeding process involved conventional breeding, together with anther culture and double hybridisation. The first generation of NERICA, varieties 1 to 11, were developed by crossing an existing *O. glaberrima* variety, CG 14, to an upland *O. sativa* variety (WAB-56-104), belonging to the japonica sub-species. NERICA varieties 12 to 18 were developed by crossing CG 14 and *O. sativa* varieties WAB-56-50 and WAB-181-181. NERICA varieties 1-18 are adapted to upland rain-fed ecologies. Furthermore, 60 NERICA varieties, NERICA 1-60, were developed for lowland agro-ecological zones. NERICA varieties vary widely in their agro-physiological traits, but are generally high yielding, early maturing, resistant to lodging and shatter (Africa Rice Centre (WARDA)/FAO/SAA, 2008).

NERICA lines of rice were introduced to farmers in West Africa in 1996 through participatory breeding approaches. By 2013, a total of 18 NERICA varieties had been disseminated to farmers in several SSA countries, including Nigeria, Sierra-

Leone, Gambia, Ghana, Guinea, Mali, Cote d'Ivoire and Togo (Adedeji *et al.,* 2013).

In Kenya, Basmati 370 is the most popular variety and accounts for 80% of rice produced in Kenya with most production being undertaken at the Mwea irrigation scheme. The variety is consumer-preferred and fetches a premium price and therefore a higher return on investment for farmers (Kihoro *et al.,* 2013). Breeding work to develop Basmati varieties were initiated in 1920s in Kala Shah Kaku (currently Pakistan). This culminated in release of the first Basmati variety, Basmati 370, in 1933. Since then, this variety has dominated the international rice trade and is considered as the standard export variety. Basmati 370 has been utilised as a pedigree for most other Basmati varieties that have been subsequently developed (Rani, 2006).

Table 4-2. Characteristics of rice varieties grown in Kenya

| Variety | Yield potential | Morphological characteristics | Maturity (Days) | Ecology | Cooking Quality | Resistance to pests |
|---|---|---|---|---|---|---|
| NERICA 1 | 4.5 tonnes $ha^{-1}$ | Height: 100 cm<br>Good resistance to lodging<br>Good tillering,<br>Colour of basal leaf sheath: purple<br>Leaf angle: erect Flag leaf angle: erect<br>Compact panicle, Grain: medium size, awning present | 95-100 | Upland rainfed | Aromatic, good | Resistance to leaf blast: medium<br>Resistance to insect pests: good |
| NERICA 4 | 5 tonnes $ha^{-1}$ | Height: 120 cm<br>Good resistance to lodging<br>Good tillering,<br>Colour of basal leaf sheath: Green<br>Leaf angle: erect Flag leaf angle: erect<br>Compact panicle,<br>Grain: Long size, Awning: absent | 95-100 | Upland rainfed | Non-aromatic; Good | Resistance to leaf blast: medium<br>Resistance to insect pests: good |

| | | | | | | |
|---|---|---|---|---|---|---|
| NERICA 10 | 6 tonnes $ha^{-1}$ | Height: 110 cm<br>Resistance to lodging: Medium<br>Good tillering,<br>Colour of basal leaf sheath: Green<br>Leaf angle: erect Flag leaf angle: erect<br>Compact panicle, Grain: medium size<br>Awning: present | 95-100 | Upland rainfed | Aromatic during flowering stage; Good | Resistance to leaf blast: good<br>Resistance to insect pests: good |
| NERICA 11 | 7 tonnes $ha^{-1}$ | Height: 105 cm<br>Resistance to lodging: Medium<br>Good tillering,<br>Colour of basal leaf sheath: Light Green<br>Leaf angle: erect Flag leaf angle: erect<br>Compact panicle, Grain: medium size<br>Awning: present | 75-85 | Upland rainfed | Aromatic during flowering stage; Good | Resistance to leaf blast: good<br>Resistance to insect pests: good |

| | | | | | | |
|---|---|---|---|---|---|---|
| Basmati 370 | 2.8 tonnes $ha^{-1}$ | Height: 145 cm<br>Susceptible to lodging<br>poor tillering,<br>Compact panicle,<br>Grain: Long and slender<br>Awning: present | 122 | Irrigated | Aromatic; Very good | Susceptible to blast<br>Resistant to rice yellow mosaic virus (RYMV) |
| BW196 | 8 tonnes $ha^{-1}$ | Height: 68 cm<br>Resistant to lodging<br>Good tillering,<br>Compact panicle, | 135 | Irrigated | fair | Resistance to blast: good<br>Susceptible to RYMV |
| IR2793-80-1 | | Height: 89 cm<br>Resistant to lodging<br>Good tillering,<br>Compact panicle, | 142 | Irrigated | Good | Resistance to blast: good<br>Susceptible to RYMV |
| ITA310 | 6 tonnes $ha^{-1}$ | Height:<br>Resistant to lodging<br>Good tillering,<br>Compact panicle, | Late maturing | Irrigated | Good | Susceptible to blast:<br>Susceptible to RYMV |

| | | | | | | |
|---|---|---|---|---|---|---|
| Duarado precoce | 5.5 tonnes $ha^{-1}$ | Height: 127<br>Resistant to lodging<br>Good tillering,<br>Compact panicle | 116 | Upland rainfed | Good | Tolerant to blast and RYMV |

Sources: Rani *et al.* 2006; Atera *et al*. 2011; Africa Rice Centre (WARDA)/FAO/SAA 2008; Ringera, 2014, KALRO, Rice knowledge bank. http://www.kalro.org/ricebank/index.php/home/rice-regions/41-rice-regions/varieties.Accessed 30-09-2017.

The most effective way of managing rice blast disease is by deployment of cultivars that are durably resistant. The pyramiding of resistance genes is therefore one strategy that can be employed to develop durable rice blast resistant cultivars (Fukuoka *et al.,* 2015; Koide *et al*., 2010). This involves introgressing more than one resistance gene into a commercial rice cultivar to offer resistance to a broad spectrum of pathotypes. However, to ensure that the resistance genes are stacked into commercial varieties effectively, it is vital to understand the population structure of the pathogen and, in particular, the most prevalent pathotypes within a given region. Limited information is available on the virulence diversity and mating type distribution of *M. oryzae* isolates from all the rice growing regions in Kenya. Furthermore, there is no documented information comparing the response of varieties commercially grown by farmers in Kenya to local population of *M. oryzae*. This study aims at filling this information gap and provide critical information for rice blast management in Kenya.

## 4.4 Materials and methods

The diversity in virulence of Kenyan isolates was undertaken, as described in section 2.2.

## 4.5 Results

### 4.5.1 Assessment of virulence diversity of Kenyan *M. oryzae* isolates

. We assessed virulence diversity of 41 *M. oryzae* isolates collected from rice growing areas in Kenya and 1 isolate, Guy 11, collected from French Guiana (Table 4-3) on a set of rice blast differential lines containing 24 R genes described above. The isolates tested represented the major lineages in Kenya, as identified in this study. Each isolate showed a variable response among the differential lines (Table 4-4). No isolate was virulent or avirulent on all the monogenic lines, indicating that the differential set was sufficient to resolve the pathotype diversity

of Kenyan isolates of *M. oryzae*. Isolates within the central Kenya lineage (KL2) showed a closely related virulence pattern, differing only on a few monogenic lines. Similarly isolates within coastal and western Kenya lineages (KL1, KL3, KL4 and KL5) had a closely related virulence spectrum (Table 4-4).

Based on the virulence diversity on the monogenic lines, cluster analysis of the *M. oryzae* isolates separated the strains into 2 major clades (Figure 4-1). With the exception of only one isolate, KE0330, clade 1 consisted of isolates from Central Kenya (the Mwea irrigation scheme). Clade 2 consisted of isolates from Coastal and Western Kenya (Kwale, Ahero and Homa-Bay). Clade 1 comprises isolates belonging to Central Kenya lineage, KL2, whereas Clade 2 is comprised of Coastal and Western Kenya lineages KL1, KL3, KL4 and KL5. The response of the monogenic lines to Kenyan *M. oryzae* isolates was analysed separately and jointly for the two clades that broadly correspond to the two of the major lineages, the Central Kenya lineage and the Coastal/Western lineage. Cluster analysis suggested that the monogenic lines fell into two broad groups based on their response but each group always comprised both resistant and susceptible rice lines (Figure 4-1).

### 4.5.2 Response of monogenic rice lines to Central Kenya lineage of *M. oryzae*

Monogenic lines harbouring the blast resistance genes *Piz5*, *Pia* and *Pi9* conferred resistance to the entire Central Kenya lineage. All other monogenic lines showed varying degrees of susceptibility (Figure 4-2). Monogenic lines harbouring *Pik* alleles (*Pik, Pik-p* and *Pik-m*), *Piz-t*, *Pi3*, *Pi7*, *Pita*2 and the susceptible control LTH were susceptible to all the isolates in the lineage. However, the susceptible control rice line, Co39, showed some resistance to a

sub-set of isolates in this lineage. Monogenic lines harbouring *Piz, Pib, Pi20, Pit* and *Pi12* showed moderate level of resistance.

Table 4-3. List of pathotyped *M. oryzae* isolates from Kenya

| Isolate | Variety sampled | Part of plant sampled | Location of Collection | GPS coordinates | Year Sampled |
|---|---|---|---|---|---|
| KE0002 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 38.23"/ E 37º 21' 39.68" | 2013 |
| KE0011 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0014 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0016 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.29"/ E 37º 21' 46.35" | 2013 |
| KE0017 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.14"/ E 37º 21' 46.25" | 2013 |
| KE0019 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.14"/ E 37º 21' 46.25" | 2013 |
| KE0021 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0029 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 44' 15.71"/ E 37º 22' 39.32" | 2013 |
| KE0036 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0036 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0202 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE0205 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE0210 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |
| KE0224 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE0225 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.51"/E 37º 22' 44.29" | 2014 |
| KE0227 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.51"/E 37º 22' 44.29" | 2014 |
| KE0234 | ITA 310 | Leaf | Western Kenya, Ahero irrigation scheme | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE0236 | Basmati 370 | Neck | Central Kenya, Mwea irrigation scheme | S 0º 38' 57.68"/ E 37º 22' 44.47" | 2014 |

| | | | | | |
|---|---|---|---|---|---|
| KE0242 | Basmati 370 | Collar | Central Kenya, Wanguru | S 0º 38' 57.64''/ E 37º 22' 44.53'' | 2014 |
| KE 255 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71''/ E34º 56' 18.37'' | 2014 |
| KE0313 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0318 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0332 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0337 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0340 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0368 | Saro | Leaf | Western Kenya, Maugo irrigation scheme, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0415 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0420 | Nerica 19 | Leaf | Coastal Kenya Kikoneni, Kwale | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0438 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 2.33''/E 39º 7' 29.80'' | 2014 |
| KE0450 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.35''/E 39º 7' 40.80'' | 2014 |
| KE0466 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 29.47''/E 39º 6' 57.89'' | 2014 |
| KE473 | Mbuyu | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 1.94''/E 39º 7' 30.48'' | 2014 |
| KE0478 | Supa | Leaf | Coastal Kenya Lunga Lunga, Kwale | S 4º 33' 29.47''/E 39º 6' 57.89'' | 2014 |

### 4.5.3 Response of monogenic lines to Coastal/Western Kenya lineage

The monogenic line harbouring resistance gene *Piz5* was the only R gene that conferred resistance to all isolates in Coastal/Western Kenya lineage (Figure 4-3). However, the monogenic line harbouring resistance gene *Pita2* conferred resistance to 90% of isolates in Coastal/Western Kenya lineage. Other resistance genes that conferred moderate resistance include *Pi12, Pita and Piz* that were effective in excluding 70% of isolates from the lineage. Susceptible controls LTH and CO39 and monogenic lines carrying resistance genes *Pik*, *Piz-t,* and *Pit* were susceptible to all isolates in the Coastal/Western lineage.

Comparing the two major lineages, the defining differences in pathotype was observed in monogenic lines harbouring *Pia*, *Pi9, Pita2* and *Pit*. The *Pi9*, *Pia* and *Pit* resistance genes were effective in excluding 100% and 70% of isolates in Central Kenya lineage, respectively, but the genes were not effective in excluding isolates from the Western and Coastal lineage. The monogenic line harbouring *Pit* was susceptible to all isolates in this lineage too. The *Pi9* and *Pia* genes conferred resistance to 45% and 17% of Western/Coastal lineage isolates, respectively. Similarly, *Pita2* was not effective in conferring resistance to the Central Kenya lineage but conferred resistance to 86% of the Western/Coastal Kenya lineage.

Combining the data from the two lineages provides evidence that *Piz5* is the most effective resistance gene against all isolates tested (Figure 4-4). Other resistance genes that conferred resistance to over 50% of isolates tested include *Pi12, Piz, Pita, Pi9, Pita2* and *Pish*. Resistance genes that were effective providing resistance to less than 10% of isolates tested include *PiI, Piz-t, Pik* and *Pik-m.*

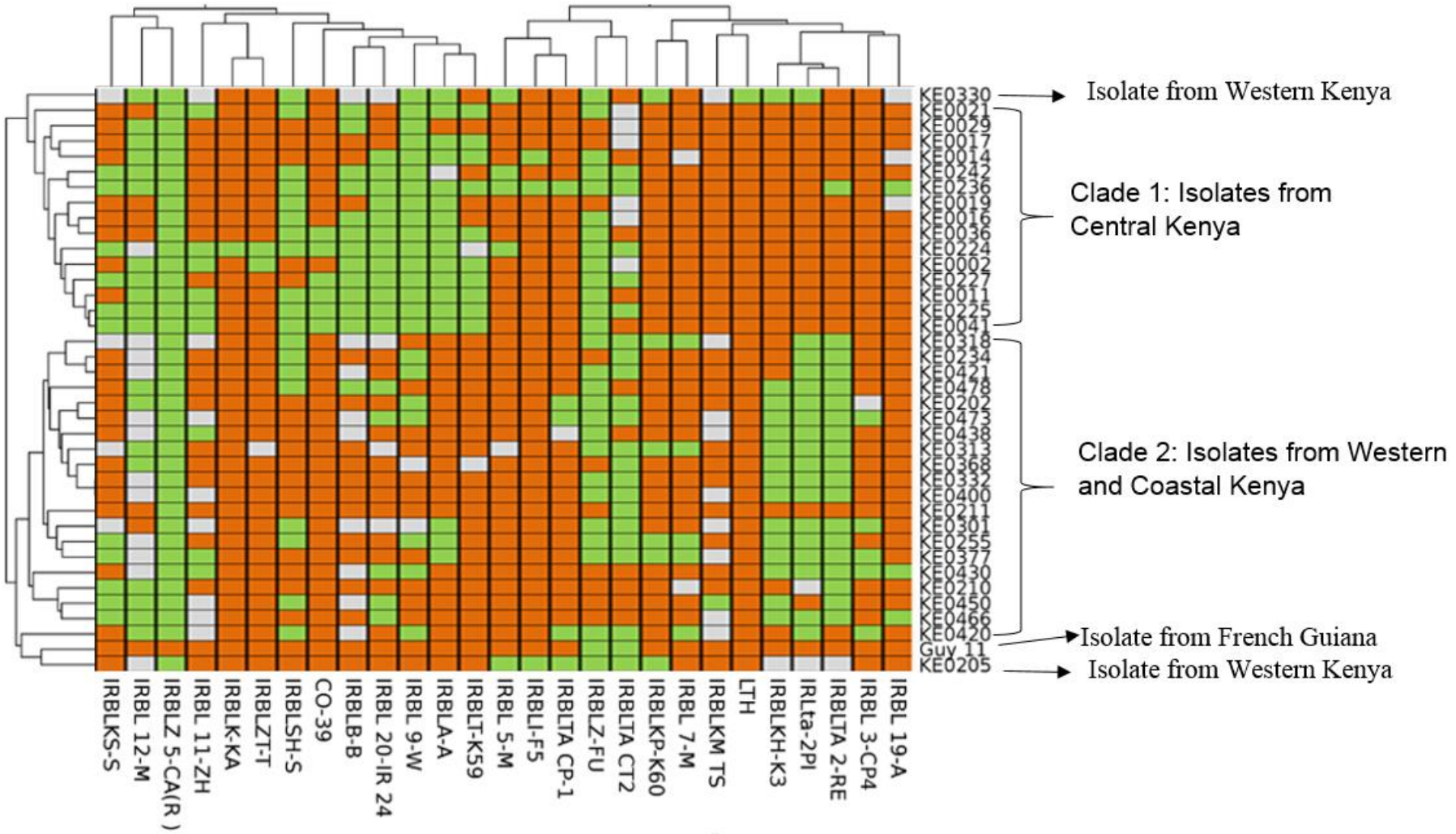


Figure 4-1. Cluster analysis showing response of rice blast monogenic differential lines to Kenyan *M. oryzae* isolates. Cluster analysis was performed by generating binary data based on response of isolates on monogenic lines where susceptible response was assigned the value 1 and resistant response assigned 0. Principal component analysis was generated using ClustVis software (Metsalu & Vilo, 2015). ■ Indicates a susceptible response, ■ indicates a resistant response, ■ monogenic line not tested.

Table 4-4. Response of rice blast monogenic lines to *M. oryzae* isolates from Kenya

| | Isolate/Lineages | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | KL1 | | KL2 | | | | | | | | | | | | | KL3 | | | | | | | | | | KL4 | | | | | | | | | | | KL5 |
| **Monogenic lines** | **Guy 11** | **KE0491** | **KE0029** | **KE0016** | **KE0017** | **KE0019** | **KE0021** | **KE0014** | **KE0002** | **KE0224** | **KE0227** | **KE0011** | **KE0036** | **KE0225** | **KE0242** | **KE0236** | **KE0255** | **KE0234** | **KE0368** | **KE0421** | **KE0430** | **KE0450** | **KE0473** | **KE0438** | **KE0415** | **KE0443** | **KE0210** | **KE0211** | **KE0205** | **KE0202** | **KE0332** | **KE0377** | **KE0313** | **KE0301** | **KE0330** | **KE0400** | **KE0478** | **KE0466** | **KE0420** |
| IRBLA-A (*Pia*) | S | S | S | R | R | R | R | R | R | R | R | R | R | R | nd | R | R | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | R | R | S | S | S |
| IRBLI-F5(*Pii*) | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S |
| IRBLKS-S (Pik-s) | S | S | S | S | S | S | S | S | S | R | R | S | S | R | R | R | R | S | S | S | S | R | S | S | S | S | R | S | S | S | S | R | nd | nd | nd | S | S | R | S |
| IRBLK-KA(*Pik*) | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| IRBLKP-K60 (*Pik-p*) | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | R | S | S | R | R | S | R | S | S | S | S |
| IRBLKH-K3 (*Pik-k*) | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | R | S | R | R | R | R | R | R | S | S | nd | R | R | R | R | R | R | R | R | R | S |
| IRBLZ-FU (*Piz*) | R | R | S | R | S | S | R | R | R | R | R | R | R | R | R | R | R | S | S | R | S | S | R | R | S | R | S | S | R | R | R | R | R | R | R | R | R | S | R |
| IRBLZT-T (*Pizt*) | S | S | S | S | S | S | S | S | R | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | nd | S | S | S | S | S | S |
| IRBLB-B (*Pib*) | S | S | R | R | S | S | R | S | R | R | R | R | R | R | R | R | S | S | S | nd | nd | nd | nd | nd | S | R | S | S | S | S | S | S | nd | nd | S | R | S | nd |
| IRBLT-K59 (*Pit*) | S | S | S | S | R | S | R | R | R | nd | R | R | R | R | S | R | S | S | nd | S | S | S | S | S | S | nd | S | S | S | S | S | S | S | S | S | S | S | S | S |
| IRBLSH-S (*Pish*) | S | S | S | R | S | R | R | S | S | R | S | R | R | R | R | R | R | R | S | R | S | R | S | S | R | R | S | S | S | S | S | S | S | R | R | S | R | S | R |
| IRBL 3-CP4 (*Pi3*) | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | R | S | S | S | S | S | S | nd | S | R | S | R | S | S | S | S | R |
| IRBL 5-M (*Pi5*) | S | R | S | S | S | S | S | S | S | R | S | S | S | S | R | R | S | S | S | S | S | S | S | S | R | R | S | S | R | S | S | S | nd | S | R | S | S | S | S |
| IRBL 7-M (*Pi7(t)*) | S | S | S | S | S | S | S | nd | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | S | nd | S | S | S | S | R | R | S | S | S | S | S | R |
| IRBL 9-W (*Pi9(t)*) | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | nd | R | R | S | R | S | S | S | S | S | S | R | S | S | S | nd | R | S | S | S | R |
| IRBL 12-M (*Pi12* (t) | S | R | R | S | R | S | S | R | R | nd | R | R | S | R | R | R | nd | nd | R | nd | nd | R | nd | nd | S | R | R | S | nd | R | nd | nd | R | S | R | nd | R | R | R |
| IRBL 19-A (*Pi19* (t)) | S | S | S | S | S | nd | S | nd | S | S | S | S | S | S | S | R | S | S | S | S | R | S | S | S | R | S | S | S | S | S | S | S | S | S | nd | S | S | R | S |
| IRBLKM TS (*Pik-m*) | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | nd | nd | R | S | S | S | S | S | nd | S | nd | nd | nd | S | nd | nd |
| IRBL 20-IR 24 (*Pi20* (t)) | S | S | S | R | S | R | S | R | R | R | R | R | R | R | R | R | S | S | S | S | R | R | R | S | nd | R | S | S | S | S | S | S | nd | nd | nd | S | R | R | S |
| IRLTA-2PI (*Pita* 2) | S | nd | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R | R | R | R | S | R | R | nd | nd | nd | S | nd | R | R | R | R | R | R | R | R | R | R |
| IRBLTA 2-RE (*Pita* 2) | S | R | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R | R | R | R | R | R | R | R | R | R | R | S | nd | R | R | R | R | R | S | R | R | R | S |
| IRBLTA CP-1 (*Pita*) | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | R | nd | S | S | S | S | R | R | S | S | S | S | S | S | S | S | R |
| IRBL 11-ZH (Pi11*(t)*) | S | R | S | S | S | S | R | S | R | R | S | R | S | R | S | S | S | S | S | S | R | nd | nd | R | S | R | S | S | S | S | S | R | S | nd | nd | nd | S | nd | nd |
| IRBLZ 5-CA (R) (*Piz5*) | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| LTH | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S |
| CO-39 | S | S | S | S | S | S | S | S | S | R | R | R | R | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |

[1] Lineages of Kenyan *M. oryzae* isolates as defined by Pot2 DNA fingerprinting.
[2] Monogenic lines and blast resistance gene harboured by line in parenthesis.
[3] Disease scored on a scale of 0-5. Where 0-2 is classified as resistant (R) while 3-5 is classified as susceptible.
nd- Not determined.
[4].R indicates a resistance response.
[5].S indicates a susceptible response.

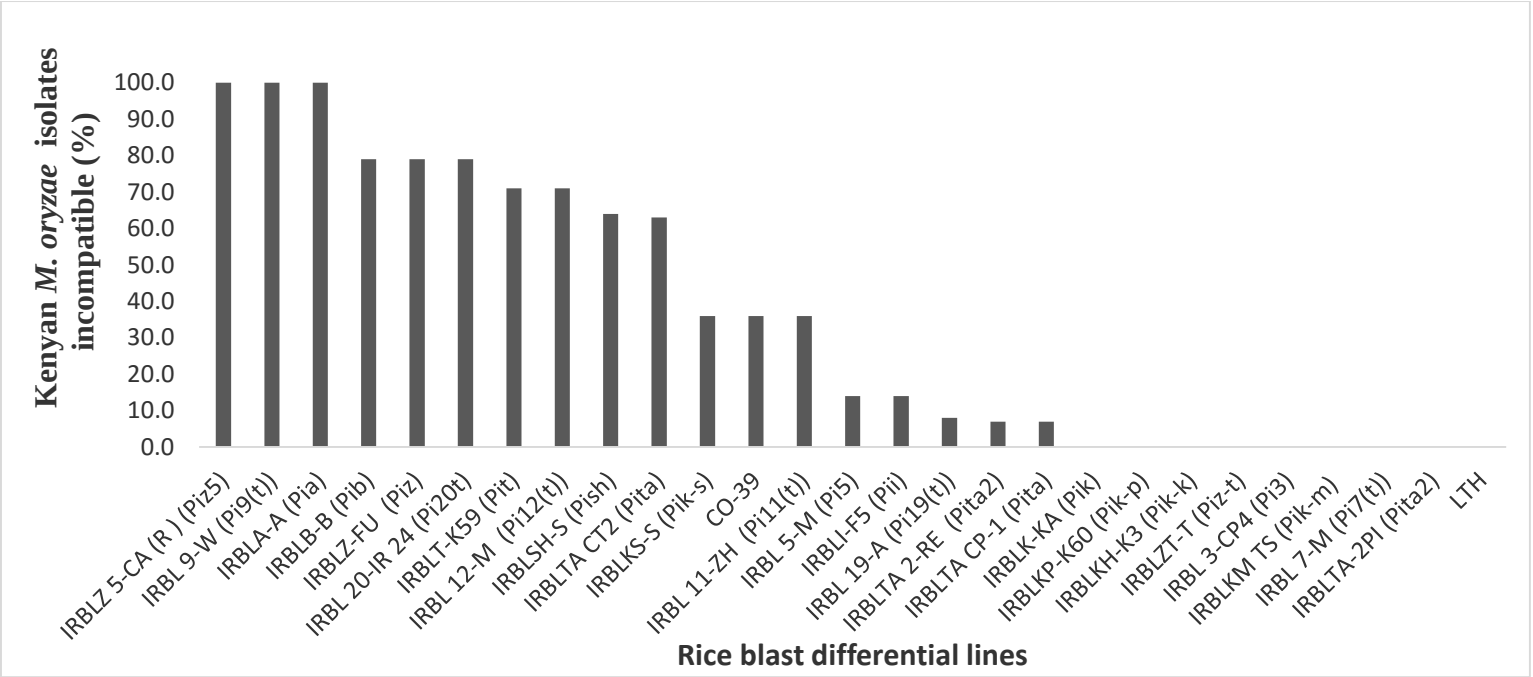


Figure 4-2. Response of monogenic rice blast differential lines to Central Kenya *M. oryzae* lineage. 21 day old monogenic lines each harbouring a single rice blast R gene were inoculated with *M. oryzae* isolates from Mwea irrigation scheme (Central Kenya) under greenhouse conditions and disease severity assessed 7 days post inoculation. Disease severity was scored on a scale of 0-5 where 0-2 is classified as resistant (R) while 3-5 is classified as susceptible.

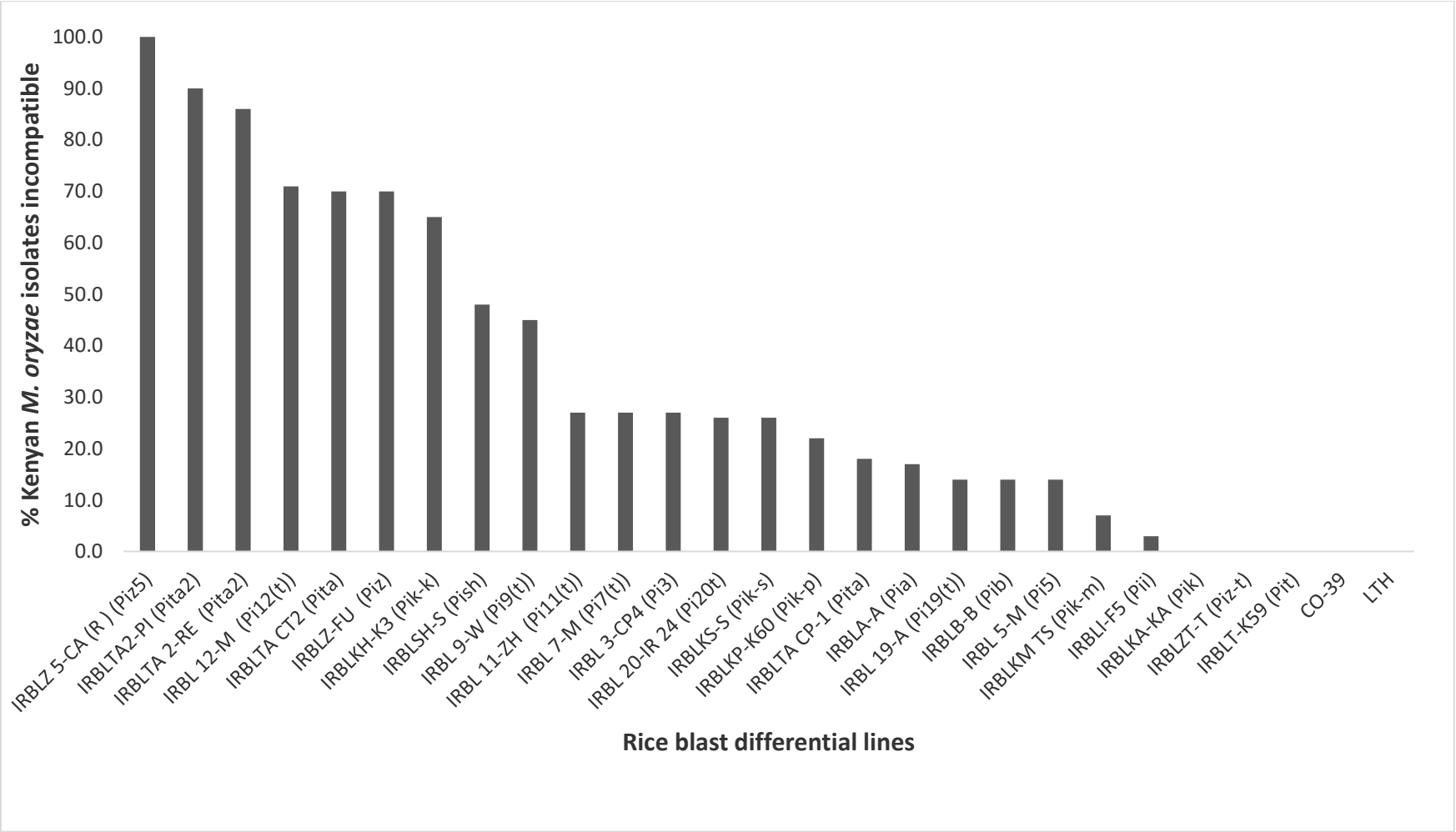


Figure 4-3. Response of monogenic rice blast differential lines to Western/Coastal Kenya *M. oryzae* lineages. 21 day old monogenic lines each harbouring a single rice blast R gene were inoculated with *M. oryzae* isolates from Ahero, Homa-Bay and Kwale (Western and coastal Kenya) under greenhouse conditions and disease severity assessed 7 days post inoculation. Disease severity was scored on a scale of 0-5. Where 0-2 is classified as resistant (R) while 3-5 is classified as susceptible.

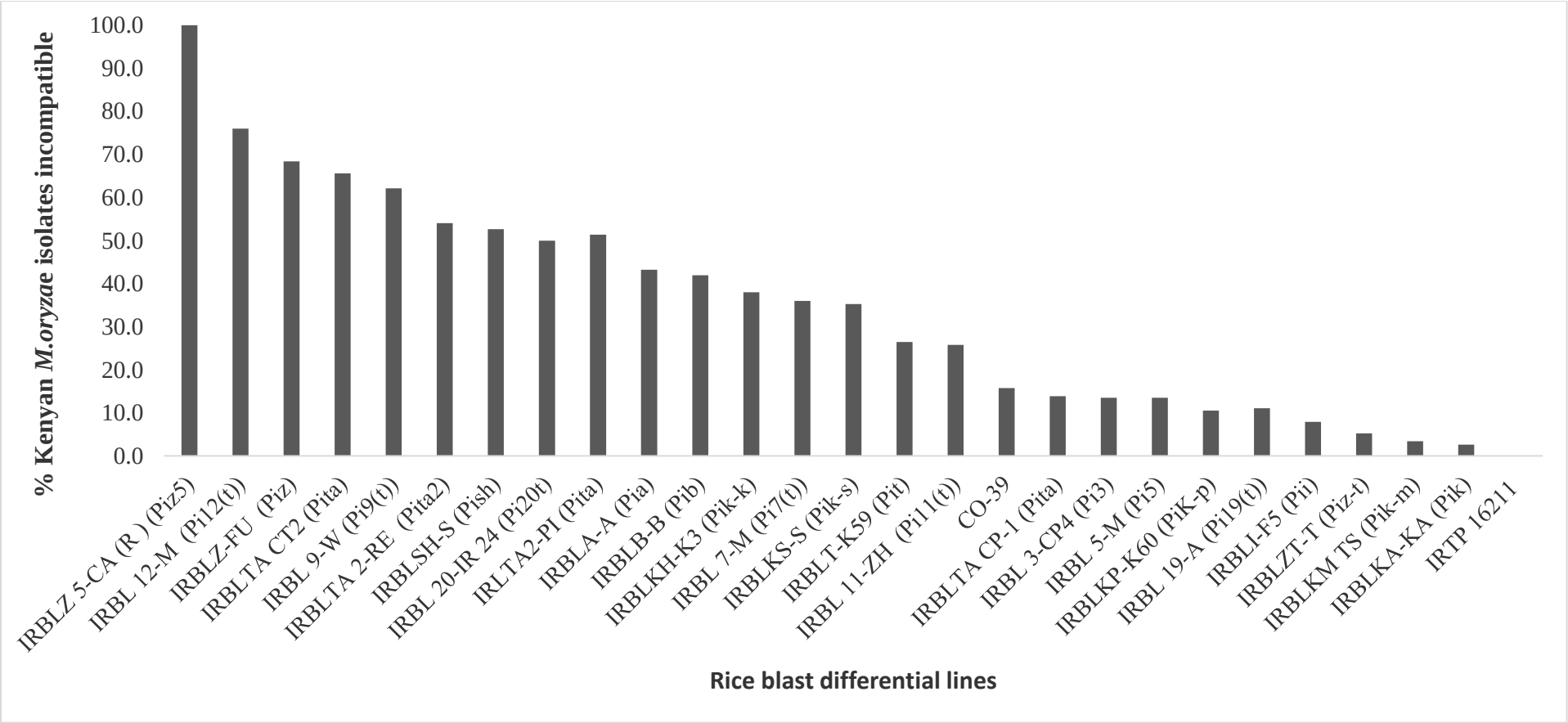


Figure 4-4. Overall response of monogenic lines to all Kenyan *M. oryzae* lineages. 21 day old monogenic lines each harbouring a single rice blast R gene were inoculated with M. oryzae isolates from all rice growing in Kenya Mwea, Ahero, Homa-Bay and Kwale (Central, western and coastal Kenya) under greenhouse conditions and disease severity assessed 7 days post inoculation. Disease severity scored on a scale of 0-5. Where 0-2 is classified as resistant (R) while 3-5 is classified as susceptible.

### 4.5.4 Response of rice varieties commercially cultivated to Kenyan *M. oryzae* isolates

The resistance/susceptibility response of Kenyan commercially grown rice varieties inoculated with Kenyan *M. oryzae* isolates was performed, as described in section 2.3.

### 4.5.5 Response of rice varieties commercially cultivated to Central Kenya lineage

Analysis of variance (ANOVA) analysis showed that rice blast severity was significant different between the varieties ($p<0.001$) (Table 4-5). Mean separation using the Bonferroni test indicated that there was no significant difference between the susceptible control, LTH, and Basmati 370 ($p=1.00$) (Table 4-6) which recorded the highest mean disease scores at 8.33 and 8.67, respectively (Table 4-7). Basmati 370 showed a significantly higher disease severity than Duarado precoce, IR2793-80-1 ITA 310, Nerica 1, Nerica 10, Nerica 11, and Nerica 4 ($p=0.001$) (Table 4-6). These varieties were predominantly more resistant to rice blast and recorded disease scores of less than 3.65 (Table 4-7). Significant differences were also observed, however, within the resistant varieties. For instance, Duarado precoce had a significantly higher mean disease score than IR2793-80-1 ($p=0.004$), ITA 310 ($p=0.001$), NERICA 11 ($p=0.004$) and NERICA 4 ($p=0.004$).

In response to rice blast infection, the varieties can be classified in the following decreasing order of resistance, which is statistically supported ($P<0.001$):

ITA310/NERICA10/NERICA11/IR2793-80-1/NERICA4/BW196>NERICA1/Duarado precoce>/LTH/Basmati 370.

Table 4-5. One-way analysis of variance of response of Kenyan rice varieties to Central Kenya *M. oryzae* lineage

| Source | Sum of Squares | Degrees of freedom | Mean square | F | Probability > F |
|---|---|---|---|---|---|
| Between groups | 287.37 | 9 | 31.93 | 45.61 | <0.001 |
| Within groups | 14 | 20 | 0.7 | | |
| Total | 301.37 | 29 | | | |

Table 4-6. Pairwise mean comparison of response of Kenyan rice varieties to Central Kenya *M. oryzae* lineage

| | BW196 | Basmati 370 | Duarado precoce | IR2793-80-1 | ITA 310 | LTH | Nerica 1 | Nerica 10 | Nerica 11 |
|---|---|---|---|---|---|---|---|---|---|
| **Basmati 370** | 7.67** | | | | | | | | |
| **Duarado precoce** | 2.67* | -5.00** | | | | | | | |
| **IR2793-80-1** | -0.67ns | -8.33** | -3.33* | | | | | | |
| **ITA 310** | -1.00ns | -8.67** | -3.67** | -0.33ns | | | | | |
| **LTH** | 7.33** | -0.33ns | 4.67** | 8.00** | 8.33** | | | | |
| **Nerica 1** | 2.33ns | -5.33** | -0.33ns | 3.00* | 3.33* | -5.00** | | | |
| **Nerica 10** | 0.00ns | -7.67** | -2.67* | 0.67ns | 1.00ns | -7.33** | -2.33 ns | | |
| **Nerica 11** | -0.67ns | -8.33** | -3.33* | 0.00ns | 0.33ns | -8.00** | -3.00* | -0.67ns | |
| **Nerica 4** | 0.00ns | -7.67** | -2.67* | 0.67ns | 1.00ns | -7.33** | -2.33ns | 0.00ns | 0.67ns |

Pairwise mean comparison using the Bonferroni test; mean differences shown; * shows mean is significantly different at p< 0.05; ** shows mean is significantly different at p< 0.001. ns shows statistically not significant.

Table 4-7. Mean responses of Kenyan rice varieties to Central Kenya *M. oryzae* lineage

| Variety | Mean[1] | Standard Error | 95% confidence interval | |
|---|---|---|---|---|
| | | | Lower limit | Upper limit |
| **Basmati 370** | 8.67 | 0.33 | 7.23 | 10.10 |
| **LTH** | 8.33 | 0.33 | 6.90 | 9.77 |
| **Duarado precoce** | 3.67 | 0.33 | 2.23 | 5.10 |
| **Nerica 1** | 3.33 | 0.33 | 1.90 | 4.77 |
| **BW 196** | 1.00 | 0.58 | -1.48 | 3.48 |
| **Nerica 10** | 1.00 | 1.00 | -3.30 | 5.30 |
| **Nerica 4** | 1.00 | 0.58 | -1.48 | 3.48 |
| **IR2793-80-1** | 0.33 | 0.33 | -1.10 | 1.77 |
| **Nerica 11** | 0.33 | 0.33 | -1.10 | 1.77 |
| **ITA 310** | 0.00 | 0.00 | 0.00 | 0.00 |

[1].Rice blast severity assessed 7 days post inoculation on a disease scale 0-9 where 0-3 was rated resistant and 4-9 rated as susceptible. Disease mean from 3 replications. .

### 4.5.6 The response of commercially cultivated rice varieties to infection by isolates of the Western and Coastal Kenya lineage

ANOVA analysis indicates that there were significant differences between rice varieties in response to the Coastal/Western Kenya lineage of *M. oryzae* (p=<0.001), as shown in Table 4-8. Mean separation using the Bonferroni test shows that there was no significant difference in mean disease score between Basmati 370 and LTH (p=1.00) and ITA 310 (p=0.3) (Table 4-9), with both showing severe disease symptoms. Disease severity in Basmati 370 and LTH was significantly higher than NERICA 1, NERICA 4, NERICA 10, NERICA 11, IR 2780-1 and Duarado precoce. The LTH, Basmati 370 and ITA 310 cultivars were susceptible to isolates of the Coastal and Western Kenya lineage and recorded disease mean scores of 7, 6 and 4.67, respectively. Duorado precoce recorded a mean disease score of 2.67 and was marginally susceptible (Table 4-10). Basmati 370 showed significantly higher mean disease score than NERICA 1 (p=0.008), NERICA 4 (p=0.004), NERICA 10 (p=0.004) and NERICA 11 (p>0.001). There was no significant differences in the disease mean score between NERICA 1, NERICA 4, NERICA 10 and NERICA 11 (p=<0.001). These NERICA varieties were resistant to rice blast and recorded mean disease scores of 1.33, 1.00, 1.00 and 0.00 respectively (Table 4-10).

In summary, in response to rice blast, the varieties can be classified in the following decreasing order of blast resistance:

NERICA1/NERICA4/NERICA10/NERICA11/IR2793-80-1/Duorado precoce>Basmati 370/LTH/ITA310.

Table 4-8. One-way analysis of variance of response of Kenyan rice varieties to Coastal and Western Kenya *M. oryzae* lineages

| Source | Sum of Squares | Degrees of freedom | Mean square | F | Probability > F |
|---|---|---|---|---|---|
| Between groups | 166.30 | 9 | 18.48 | 12.05 | <0.001 |
| Within groups | 30.67 | 20 | 1.53 | | |
| Total | 196.97 | 29 | 6.80 | | |

Table 4-9. Pairwise mean comparison of response of Kenyan rice varieties to Coastal and Western Kenya *M. oryzae* lineage

| | BW196 | Basmati 370 | Duarado precoce | IR2793-80-1 | ITA 310 | LTH | Nerica 1 | Nerica 10 | Nerica 11 |
|---|---|---|---|---|---|---|---|---|---|
| **Basmati 370** | 4.33** | | | | | | | | |
| **Duarado precoce** | 0.00ns | -4.33** | | | | | | | |
| **IR2793-80-1** | -2.67ns | -7** | -2.66ns | | | | | | |
| **ITA 310** | 2.00ns | -2.33ns | 2.00ns | 4.67** | | | | | |
| **LTH** | 4.33** | 0.00ns | 4.33** | 7.00** | 2.33ns | | | | |
| **Nerica 1** | -1.33ns | -5.67** | -1.33ns | 1.33ns | -3.33* | -6.67** | | | |
| **Nerica 10** | -1.67ns | -6.00** | -1.67ns | 1.00ns | -3.67* | -6.00** | -0.33ns | | |
| **Nerica 11** | -2.67ns | -7.00** | -2.67ns | 0.00ns | -4.67** | -7.00** | -1.33ns | -1 ns | |
| **Nerica 4** | -1.67ns | -6.00** | -1.67ns | 1.00ns | -3.67* | -6.00** | -0.33ns | 0.00ns | 1ns |

Pairwise mean comparison using Bonferroni test; mean differences shown; * shows mean is significantly different at p< 0.05; ** shows mean is significantly different at p<0.001. ns shows not statistically different.

Table 4-10. Mean responses of Kenyan rice varieties to Western and Coastal Kenya *M. oryzae* lineages

| Variety | Mean[1] | Standard Error | 95% confidence interval | |
|---|---|---|---|---|
| | | | Lower limit | Upper limit |
| **LTH** | 7.00 | 1.00 | 2.70 | 11.30 |
| **Basmati 370** | 7.00 | 0.58 | 4.51 | 9.48 |
| **ITA 310** | 4.67 | 0.33 | 3.23 | 6.10 |
| **Duarado precoce** | 2.67 | 0.33 | 1.23 | 4.10 |
| **BW 196** | 2.67 | 0.33 | 1.23 | 4.10 |
| **Nerica 1** | 1.33 | 0.88 | -2.46 | 5.13 |
| **Nerica 10** | 1.00 | 0.58 | -1.48 | 3.48 |
| **Nerica 4** | 1.00 | 0.58 | -1.48 | 3.48 |
| **IR2793-80-1** | 0.33 | 0.33 | -1.10 | 1.77 |
| **Nerica 11** | 0.00 | 0.00 | 0.00 | 0.00 |

## 4.6 Discussion

Various mechanisms have been proposed to contribute to genotype and pathotype variation in asexual populations of *M. oryzae*, including parasexual reassortment and recombination, and mutations in *AVR* genes arising from deletions, substitutions, insertion of transposons, or larger-scale genomic rearrangements, such as reciprocal translocations, chromosome breakage and mini-chromosome generation (Farman *et al.,* 2002; Orbach *et al.,* 2000; Zeigler *et al.,* 1997). In this study, we have shown that a complex relationship exists between the identified lineages and pathotypes, with lineages comprising of multiple pathotypes, but with closely related virulence spectra. These findings are consistent with other studies that have reported a complex relationship between lineages and pathotypes in most rice-growing regions worldwide (Chipili, 2000). An analysis of the genetic and virulence diversity of isolates from 4 countries in sub-Saharan Africa, including Ghana, Burkina Faso, Nigeria, Cote d'Ivoire reported that there were 1-12 pathotypes per lineage, with the majority of lineages containing multiple pathotypes (Chipili, 2000). In the United States, Xia *et al.* (1993) analysed a collection of 130 *M. oryzae* isolates from two commercial rice fields in Arkansas and confirmed the existence of seven of the eight lineages identified in an earlier study by Levy *et al.* (1991). The lineages, however, showed greater heterogeneity in their virulence than in the earlier study, with multiple pathotypes occurring within lineages. Similar findings have been documented for *M. oryzae* populations in Philippines (Zeigler *et al.,* 1995). Taken together, these findings indicate that in most rice-growing regions, clonal lineages consist of isolates that are closely related, genetically, but still with some heterogeneity in pathotype. Changes in virulence may occur more rapidly than genomic changes that lead to divergence of clonal lineages.

Zeigler *et al.* (1995) proposed that a clonal lineage is the basic informative unit for understanding *M. oryzae*-host interactions. He argued that first, a large number of isolates and pathotypes can be reduced to a few distinct groups and secondly, that this enables one to observe any promiscuous changes within a clonal lineage in a more specific manner than had occurred previously. In this case, one may, for instance, observe both virulent and avirulent isolates on given hosts. This also suggests that some incompatible interactions may be unstable. Using a clonal lineage as the basic unit of analysis of rice blast in a population, allows the researcher to generate a composite view of lineage-host interactions. Some resistance genes, for example, confer resistance to an entire lineage, which provides evidence that it may be more difficult for the lineage to overcome resistance conferred by such R genes. R genes excluding the entire lineage are therefore more suitable candidates for breeding for rice blast resistance, especially when they can be used in combinations. In this study, I observed that the R gene *Piz5* conferred resistance to all Kenyan *M. oryzae* lineages. The R genes *Pi9* and *Pia* conferred resistance to the entire central Kenya lineage and although the *Pita2* R gene did not confer resistance to the entire Coastal/Western Kenyan lineage, it did exclude 90% of isolates in this lineage. This combination of genes therefore provides the basis for a durable disease control strategy.

Lineage exclusion breeding strategy has been proposed as a strategy for generating durable rice blast resistance (Zeigler *et al.,* 1994). This strategy relies on pyramiding several major R genes into a single cultivar to exclude an entire virulence spectrum in a population of *M. oryzae*. The lineage exclusion strategy has been successfully utilised to develop rice blast resistant lines. In India, pyramiding *Pi1* and *Pi2* R genes in Co39 was sufficient to exclude all the 29 *M. oryzae* lineages identified and confer resistance to Co39 in rice blast disease

hotspots. Recently, Xiao *et al.* (2017) demonstrated that by pyramiding *Pi9* or *Piz-t* and *Pi54* in a rice blast susceptible variety, 07GY31, led to improved leaf and panicle blast resistance.

Our studies indicate that *Piz5*, *Pita2* or *Pi9* are suitable candidates for pyramiding into the rice blast susceptible variety, Basmati 370, to confer resistance against the 5 identified lineages in Kenya. As a part of this project, these R genes have recently been reported to have broad resistance spectrum on SSA *M. oryzae* isolates (Mutiga *et al.,* 2017), consistent with an earlier report (Odjo *et al.,* 2014). Additionally, rice blast resistance genes *Pi9* and *Piz5* have been cloned, and microsatellite markers linked to these rice blast genes have been developed (Sharma *et al*., 2012; Miah *et al*., 2013) and can therefore be used in a marker assisted (MAS) breeding strategy. Molecular marker assisted plant breeding (MAB) involves application of molecular techniques to improve plants on the basis of genotypic assays. This includes several breeding strategies *viz*. marker-assisted selection (MAS), marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS) and genome-wide selection (GWS) (see Liang, 2015). Incorporation of MAS into rice blast breeding will accelerate the breeding process and hasten the availability of resistant varieties to farmers. A recent study has examined the virulence spectrum of *M. oryzae* isolates in Kenya. Although some findings are consistent with our study, some discrepancies are also noted. Nyongesa *et al.* (2016) reported, contrary to our study, that monogenic lines harbouring R genes *Piz-t, Pik-s, Pik-p, Pik-h* and *Pik* were resistant to rice blast disease under natural infections. The discrepancies may be accounted for by several factors including: (1) differences in inoculation method (natural versus laboratory inoculation) leading to variations in inoculum density and distribution. (2) differences in genetic background of rice lines used. In our

study, we utilised monogenic lines with R genes introgressed in the LTH genetic background whereas in Nyongesa *et al.* (2016), monogenic lines with both LTH and Co39 backgrounds were used. In their study, variations in response of lines with LTH and Co39 backgrounds were also observed in the same locality. This was attributed to differences in presence of unknown rice blast resistance genes in LTH and Co39. (3) differences in the environmental conditions between the two experiments. (4) differences in score scales and disease assessment. (5) differences in the geographical locations between the experiment sites and our isolate collection. (6) variations due to evolution of avirulence genes in the population.

In spite of the potential to lead to durably disease-resistant cultivars, pyramiding of resistance genes should be approached with caution because it does not always lead to enhanced rice blast resistance. For example, Xiao *et al.* (2017) showed that lines pyramided with *Pi9* and *Pi54* exhibited lower resistance levels than *Pi9* monogenic lines. Similarly, results by Tabien *et al.* (2000) indicate that a stack of four resistance genes was less effective compared to a stack of three resistance genes. These studies show that gene pyramiding may result in negative interactions, leading to reduced rice blast disease resistance and therefore the effectiveness of gene pyramiding for rice blast resistance needs be assessed on a case by case basis with careful field testing in disease hotspots nurseries.

Our study indicates that Basmati 370 is susceptible to all rice blast isolates tested and recorded disease a score of >6, on a scale of 0-9, to all Kenyan *M. oryzae* lineages identified in this study. The variety Duorado precoce was moderately susceptible with NERICA 1, NERICA 4, NERICA 10, NERICA 11, IR2993-80-1 and BW 196 showing resistance to all the lineages. Differences in response of

ITA 310 to *M. oryzae* lineages were also observed. The variety is resistant to the Central Kenya lineage, but susceptible to the Western/Coastal Kenya lineage of *M. oryzae* isolates. The variety is predominantly cultivated in Western Kenya and therefore the *M. oryzae* population in this region may have evolved to gain virulence on this variety. Our findings are consistent with those of Kihoro *et al.* (2013) which reported high rice blast incidence in Basmati 370 at the Mwea irrigation scheme. Rice blast resistance in NERICA lines has been reported and may be attributed to the *O. glaberrima* (African rice) background (Mgonja *et al.,* 2017; Mutiga *et al.,* 2017; Africa Rice Centre (WARDA)/FAO/SAA, 2008). *O. glaberrima* originates from West Africa where it has been known to be cultivated for more than 3,500 years and therefore has developed adaptive mechanisms to both biotic and abiotic stresses (Jones *et al.,* 1997).

In Kenya, Basmati rice accounts for 80% of rice produced and is mainly cultivated in Mwea irrigation schemes as a monocrop (Kihoro *et al.,* 2013). This cropping system involves a monoculture of a single susceptible variety which creates a conducive environment for rice blast epidemics in the irrigation scheme. A few farmers co-cultivate Basmati 370 with the rice blast resistant variety BW196. However, BW 196 is not a consumer preferred variety and therefore its cultivation is limited. Recycling of resistance genes has been suggested as a strategy for disease management, including under circumstances in which some resistance genes have been overcome. The gain of virulence in pathogens may be associated with a loss of fitness of the pathogen. In such situations, co-cultivation of both susceptible and resistance varieties reduces possibilities of disease epidemics. In addition, it ensures efficient utilisation of resistance genes and has an economic benefit because each resistance gene costs significant resources to breed and release to farmers (Howlett *et al.,* 2015).

Compared to other rice varieties, Basmati 370 is considered agronomically inferior in some respects. Basmati varieties are, for example, generally low yielding, tall, susceptible to lodging and respond poorly to fertilisation as well as being susceptible to rice blast disease (Rani *et al*., 2006, Kihoro *et al.,* 2013). The main reason Basmati 370 is the preferred rice variety in most rice growing regions, however, is due to its superior aroma. Due to its aroma, it attracts a premium price making its cultivation a risk worth taking for most farmers. This trend is also unlikely to change in the future. Future research efforts should, therefore, be directed towards improving the agronomic qualities of Basmati 370. Our study in collaboration with others (Mutiga *et al.*, 2017), has contributed valuable information towards breeding for rice blast resistance in Basmati 370 in East Africa. Currently, as part of our overall BBSRC/Gates/DFID/Halpin project there are on-going efforts to pyramid R genes (*Pi9, Piz5* and *Pita2*) and quantitative trait loci (*Pi35* and *Pi21*), into Basmati 370 and Basmati 217 varieties (Zhou, 2017).

An effective rice blast surveillance system is also an integral part of sustainable blast management. In collaboration with stakeholders, we have implemented a rice blast surveillance system in Kenya, involving use of plant health clinics that are strategically located in rice-growing regions. The plant health clinics were developed in Israel and first applied in South East Asia (Ausher *et al*., 1996). In Kenya, plant clinics have previously been successfully utilised to offer diagnostic services to advise on crop health management in various crops (Otipa *et al.,* 2015). These plant clinics are operated by trained plant disease diagnosticians (commonly referred to as “plant doctors”). The plant doctors are provided with reference materials, such as factsheets and photographs of crops and pests in their respective agro-ecological zones. The plant doctors liaise with referral

laboratories for any further diagnosis or analysis and therefore are an important component of rice blast surveillance and disease management. In addition, the strains collected in this study are stored as reference strains in BecA-ILRI in Nairobi where an ongoing collection service is co-ordinated from, in collaboration with KALRO.

In summary, this study provides information that will help to guide rice blast breeding activities in Kenya. Further efforts should focus on collection and screening of new isolates. This will ensure new pathotypes are detected and an appropriate breeding strategy developed.

# Chapter 5: Comparative genomic analysis of sub-Saharan rice blast isolates

## 5 Introduction

### 5.1 Genome sequencing of phytopathogens

Comparative analysis of genome sequences of phytopathogenic fungi and oomycetes has become an essential component in understanding disease biology, diagnosis and management (Klosterman *et al.,* 2016). Recently, significant progress has been made in the number of phytopathogens, for which genomes have been sequenced and this can be attributed both to the importance of genomic data in understanding phytopathogenesis, and due to the lower cost of high throughput genome sequencing (Moller & Stukenbrock, 2017; Soanes *et al.,* 2007). Large-scale genome projects have, for example, been initiated including the genome 10K (Genome 10K, Community of Scientists 2009), 5000 insect genome project (Robinson *et al.,* 2011), and 1000 plants (projects www.onekp.com) . Although fungi are the most sequenced kingdom of organisms, and a target of 1000 fungal genomes has already been achieved, the goal of having two representative genome sequenced per family has been achieved only in 85 families in the *Ascomycota*, 66 in the *Basidiomycota*, and 11 in the remainder of the kingdom Fungi. Furthermore, the genomes of 191 fungal phytopathogens, infecting a total of 171 crop species are publicly available as of 2017, and these belong primarily to the phyla *Ascomycota* and *Basidiomycota* (Aylward *et al.,* 2017). Among the 1094 publicly available fungal genomes, 41.4% represent plant and animal pathogens and other medically important fungi, such as fungi involved in food spoilage, out of which plant pathogens account for 49.4% of the species in this group (Aylward *et al.,* 2017). According to the Genomes Online Database (Bernal *et al.,* 2001), the genome sequences of 48

strains of *M. oryzae* are publicly available, second only to *Saccharomyces cerevisiae* (for which there are 166 sequenced strains).

The genome sizes of phytopathogens vary considerably both within and between the taxa, with powdery mildews, rust fungi and *P. infestans* having the largest genomes in ascomycetes, basidiomycetes and oomycetes respectively (Haas *et al.,* 2009; Spanu *et al.,* 2010; Duplessis *et al.,* 2011). The genomes of a number of plant pathogens have expanded in size in comparison to related saprotrophs and this may be attributed to the proliferation of transposable elements, which is widespread among phytopathogens (Haas *et al.*, 2009; Spanu *et al*., 2010). Larger genome sizes may also be as a result of expansion of pathogenesis-related genes. For example, in basidiomycetes and euascomycetes, duplication of gene families associated with melanin biosynthesis, host cell degradation and transport functions have been reported (Powell *et al.*, 2008). Furthermore, lineage-specific expansion of pathogenesis-related genes has been reported in oomycetes (Adhikari *et al,.* 2013). However, some other pathogens, for example, *Ustilago maydis*, *Albugo laibachii* and *Sclerotiorum sclerotinia* have relatively smaller genome sizes due to low transposon activity, but also including loss of both introns and genes (Amselem *et al.*, 2011; Kemen *et al.,* 2011; Kämper *et al.,* 2006).

Various regions within the genome are known to be over-represented in putative effector-encoding genes and they include gene-sparse regions (transposon islands), gene clusters, isochore-like regions, subtelomeric regions and conditionally dispensable chromosomes (CDCs) as reviewed by Möller & Stukenbrock, (2017).

## 5.2 Mechanisms involved in the evolution of Fungi genome

### 5.2.1 DNA Point Mutations

The mutation rate of an organism refers to the number of mutations per cell division, per generation, or per time (Baer *et al*., 2007). Natural selection affects the mutation rate per generation and acts directly on genomic mutation rate. Mutation rates due to natural selection may vary between individuals due to several factors. Firstly, differential regulation of DNA polymerases, that have varying fidelities during the DNA replication process, may occur. Secondly, differences can exist among individuals in their capacity to detect and repair DNA damage, and thirdly differences in mutation buffering capacity. In addition, variations in mutation rate have been reported between and within chromosomes in a single genome. The potential causes of variation in mutation rates within a genome are not fully understood, but are thought to be due to base composition, local recombination rates, patterns of gene expression and gene density. Some genomic regions are, for instance, prone to high levels of nucleotide substitutions. For example, Cuomo *et al.*(2007) identified 10,000 SNPs between two strains of *Fusarium graminearum*, with a majority of SNPs being located in telomeres and regions of high recombination. Some of the SNPs were identified in genes that are expressed during infection, including predicted secreted proteins, amino acid transporters and cytochrome P450s. SNPs have been involved in host shifts, as exemplified in a study by Raffaele *et al.* (2010). The authors undertook a comparative study of closely related *Phytophthora* spp. (*P. infestans, P. ipomoeae, P. mirabilis,* and *P*. *phaseoli*) that are adapted to different hosts and identified several mutations, including SNPs, copy number variations and presence/absence of genes. The authors observed higher frequency of copy number variations, presence/absence polymorphisms in genes located in the

gene-sparse regions compared to gene-dense regions. Even though the frequency of SNPs was similar across the genomes, the average dN/dS ratio was significantly higher in gene-sparse regions, indicating that more genes had signatures of positive selection.

### 5.2.2 Recombination

Meiotic recombination is an important driver of evolutionary changes in sexually reproducing organisms and plays a crucial role in genome-wide nucleotide variation patterns (Begun & Aquadro, 1992), rates of protein evolution (Begun & Aquadro, 1992), transposable element distribution (Rizzon *et al*., 2002), GC content (Meunier & Duret, 2004) and codon bias (Marais *et al.*, 2003). In sexual reproduction, pairs of homologous chromosome replicate during meiosis before undergoing reciprocal recombination (crossing over) and then segregate during meiosis I and II (Barton *et al.,* 2008). Recombination studies based on population genomic data have been performed on different species, including mammals, birds, insects, plants and fungi. Taken together, the results indicate that recombination rates vary within a genome with some regions showing high frequency of recombination and are referred to as recombination hotspots.

Subteleomeric regions have been reported as recombination hotspots in *Zymoseptoria tritici* (Croll *et al.,* 2015) and *Saccharomyces cerevisiae* among other fungi (Barton *et al.,* 2008). According to Barton *et al.* (2008), occurrence of recombination hotspots in subtelomeric regions may be attributed to telomere clustering and their ‘bouquet’ arrangement, which brings end-most homologous sequences into closer proximity than homologous sequences in other regions of the chromosome. In *Z. tritici,* recombination hotspots were associated with low linkage disequilibrium and were enriched with genes that encode for secreted proteins indicating that localisation of these genes in the hotspots was favoured

by selection pressure (Croll *et al.*, 2015; Stukenbrock & Dutheil, 2017). This suggests that, in addition to the previously described repeat-rich genome regions, recombination hotspots constitute genomic compartments which favour rapid evolution of virulence.

### 5.2.3 Repeat-induced point mutations (RIP)

Repeat induced point (RIP) mutations were originally described in *Neurospora crassa* (Selker *et al.,* 1987) and this mechanism is used by some fungi to suppress the proliferation of transposable elements in a genome (Clutterbuck, 2011). RIP mutations are characterised by the presence of repeat family sequences in which some copies can be observed to have reduced GC content and differ from each other by multiple C to T transitions. This arises by RIP and can be readily observed following meiosis. Sequence variation in transposable elements arising from RIP mutations have been described in several fungi including *M. oryzae* (Ikeda *et al.,* 2002)*, Leptosphaeria maculans* (Idnurm & Howlett, 2003) *Nectria haematococca* (Coleman *et al*., 2009) and *Colletotrichum cereale* (Crouch *et al.,* 2008). The extent of RIP mutation and dinucleotide transitions varies among species, as exemplified by a large study involving 54 genomes of 49 species belonging to ascomycete subphylum Pezizomycotina (Clutterbuck, 2011). RIP mutations in *M. oryzae* show similarity with *N*. *crassa* including (i) RIP mutations occur only during the sexual phase (ii) the transitions were found in both linked and unlinked duplicated sequences (iii) MAGGY sequences mutated by RIP showed only C-to-T or G-to-A changes on a given chain (Ikeda *et al*., 2002; Clutterbuck, 2011).

According to Raffaele & Kamoun, (2012), RIP mutations cause inactivation of genes by introducing premature stop codons or non-synonymous substitutions with the mutations having the potential to leak to regions flanking the repeat

sequences. Since avirulence effector genes are closely associated with repeat-rich regions they are therefore candidates that could be affected by RIP induced mutations.

### 5.2.4 Transposable elements

Transposable elements (TEs) were first discovered in maize in the 1940's by Barbara McClintock, but have now been identified in animals, fungi and bacteria (Finnegan, 1989). In fungi, TEs were first identified in *S. cerevisae* (Cameron *et al.*, 1979). TEs are classified based on their method of transposition and comprise of class I (retro-elements) and class II (DNA elements) (McDonald, 1993). The class I (retro-elements) TEs transcribe by reverse transcription of an mRNA intermediate. These are further subdivided into 2 groups: (i) retroelements with long terminal repeats (LTRs) that encode products with homology to the retroviral *gag*- and *pol*- encoded proteins, (ii) non-LTR retroelements, also called long dispersed nuclear element structures (LINE-like elements retroelements), that encode *gag*- and *pol*- proteins, but lack LTRs and have poly (A) tails at their 3' end. Although the short interspersed elements (SINEs) also transpose through an RNA intermediary, they are not classified as retroelements since they lack reverse transcriptase. The reverse transcriptase activities for the SINE elements have been shown to originate instead from non-LTR retrotransposons. The class II (DNA elements) transpose through a DNA intermediary and contain small inverted terminal sequences of less than 100 bp that borders the sequences encoding transposase enzyme. The LINE elements contain two long open reading frames that have similarity to *gag,* reverse transcriptase and RNase (McDonald, 1993). Recently, classification of LTR transposons has been based on order of the coding regions of structural (*gag*) and enzymatic (*pol*) proteins in which two broad families were recognised, Metaviridae (gypsy) and

Pseudoviridiae (*copia*). In the Metaviridae, the order of *pol* genes is protease/reverse transcriptase/RnaseH/integrase whereas in Pseudoviridiae the order is protease/integrase/reverse transcriptase/RnaseH (Figure 5-1). Class II consists of the following super families (i) *Tc1/mariner* (ii) *hAT* (iii) *mutator* and (iv) miniature inverted-repeat transposable elements (*MITE*), *Tc1/mariner* being the predominant type of transposon in fungi (Figure 5-1).

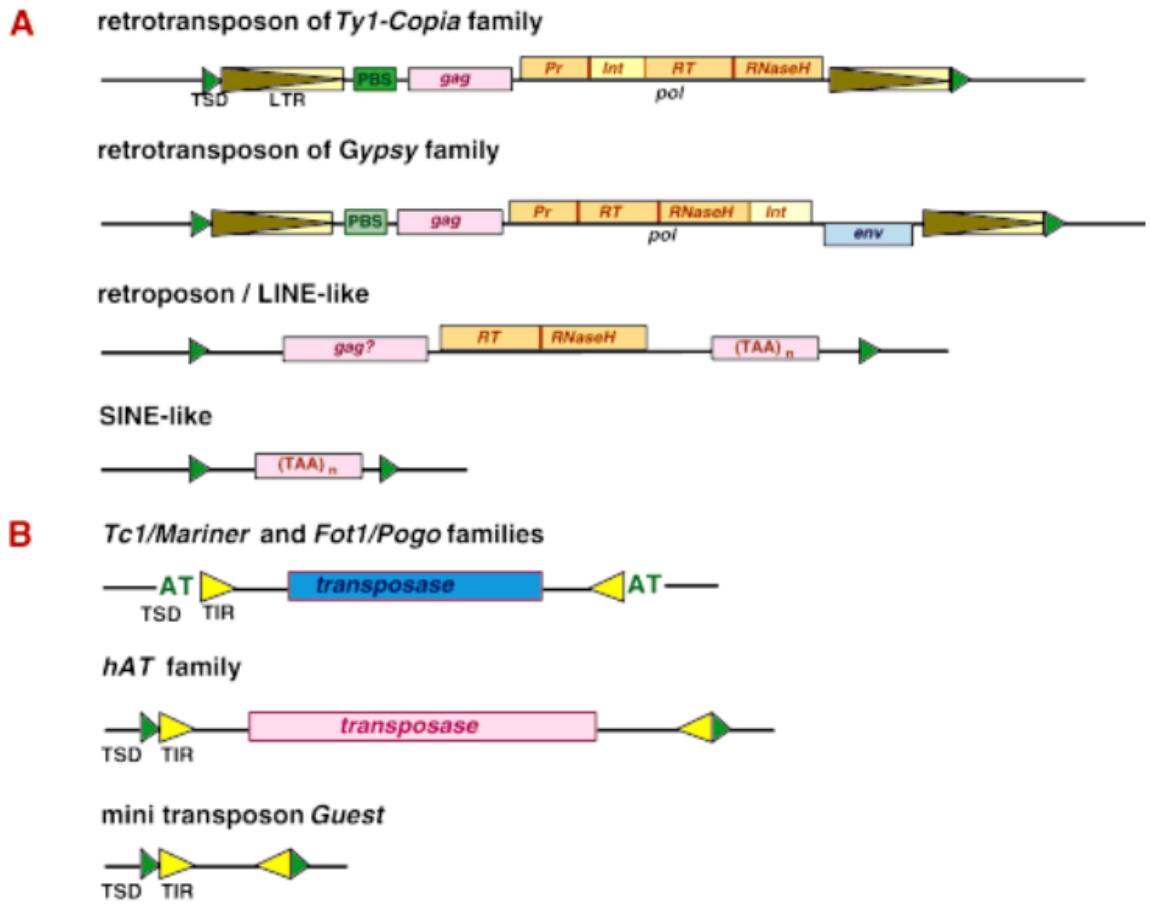


Figure 5-1. Structure and characteristics of transposable elements. A: Class I transposable elements B: Class II transposable elements. In copia elements order of pol gene is protease/integrase/reverse whereas in gypsy elements the order is protease/reverse transcriptase/RnaseH/integrase. Tc1/ elements always have 'AT' target site duplications (TSD) at the insertion site. Tc1 mariner have 'AT' at TSD but encode a different transposase. PBS, primer binding site; gag, gene for structural proteins; pol, gene for transposition; PR, protease; Int, Integrase; RT, reverse transcriptase; (TAA)n poly-A-tail. LTR, long terminal repeat; TIR, terminal inverted repeat. Source: Kempken & Kuck, (1998).

TE-mediated mutations in animals, plants, bacterial and fungi have widely been reported in literature and collectively these studies indicate that transposons are key drivers in the evolution of genomes leading to deletions (Hartmann *et al.,* 2017; Daverdin *et al*., 2012), RIP-mediated insertions (Rouxel *et al.,* 2011), chromosomal rearrangements (Hartmann *et al.,* 2017), duplications and horizontal gene flow (Richards *et al.,* 2011). In addition, TEs have been implicated in the expansion of phytopathogen genomes and lineage-specific virulence genes, as exemplified in *P. infestans* (Adhikari *et al.,* 2013). To control excessive proliferation of TEs in their genomes, fungi have evolved the mechanism of RIP, as described above. Overall these studies show that the presence of TEs in a genome is a facilitator of various mechanisms involved in genetic variation of fungi and oomycetes.

### 5.2.5 Horizontal gene transfer

Horizontal gene transfer (HGT) in fungi is thought to occur through hyphal fusion (anastomosis) resulting in the transfer of genetic material between individuals without the necessity of sexual reproduction taking place (Richards *et al.*, 2006; Morris *et al.,* 2009). A survey of 60 fungal genomes revealed that more than 700 genes of prokaryotic origin were acquired through HGT. Transfer of DNA segments or entire chromosomes been shown to confer or alter virulence of fungal pathogens. For example, in *Fusarium oxysporum*, host-specificity was acquired by horizontal transfer of four entire accessory chromosomes that harboured lineage-specific genomic regions (Ma *et al.,* 2010). Similarly, in oomycetes evolution of pathogenicity has been facilitated by inter-kingdom HGT leading to lineage-specific virulence factors (Richards *et al.,* 2006; Richards *et al.,* 2011). In *P. ramorum,* 7.6% of the secreted proteome has been acquired from fungi and includes genes that encode products involved in virulence, plant cell

wall degradation, acquisition of sugars, nucleic acids, nitrogen, and phosphate from the environment (Richards *et al.,* 2011). In *Magnaporthe oryzae,* 11 genes showed significantly higher level of sequence similarity with genes from *Phytophthora* spp. than other fungi and this was attributed to HGT (Richards *et al.,* 2006). Similarly, inter-transfer between various ascomycete and oomycete spp. has been widely reported (Jiang & Tyler, 2012).

Collectively, these studies indicate that HGT confers adaptive traits that enables fungi to acquire virulence to new host plants and adapt to new environmental conditions.

### 5.2.6 Epigenetic regulatory processes

Excessive proliferation of TEs in the genome leads to loss of fitness and therefore fungi have developed epigenetic regulation to inhibit the proliferation of these elements. TEs under epigenetic regulation retain their full capacity to self-replicate, but are silenced by a repressive chromatin environment (Slotkin & Martienssen, 2007). Epigenetic regulation of TEs results in transcriptional, or post-transcriptional modification of gene expression without changes in DNA sequences and the following process are involved: (i) post-transcriptional silencing by RNA interference (RNAi) for example in *Caenorhabditis elegans* (Sijen & Plasterk, Ronald, 2003) (ii) Inactivating chromatin by modification of histone tails, DNA methylation and changes in chromatin packing and condensation (Gendrel *et al.,* 2002).

RNAi silencing of TEs involves the formation of heterochromatin which may diffuse to the neighbouring genes thus affecting their duplication rates and expression (Vetukuri *et al.,* 2011; Hollister & Gaut, 2009).

In this chapter, I report the analysis of 27 genome sequences of *M. oryzae* originating from SSA and selected on the basis of phylogenetic and pathotype analyses reported previously. I describe my attempts to utilise these genomic sequences in order to locate and characterise novel avirulence genes in the rice blast fungus that are cognate to the R-genes that appear to be the most durable for blast control in SSA.

## 5.3 Materials and Methods

We sequenced and analysed genomes of 27 isolates from SSA, including 14 isolates collected from rice growing regions in Kenya as described in section 2.4

## 5.4 Results

A summary of sequencing statistics is shown in Table 5-1. The genome sizes ranged from 38.9-41.6 MB and with genome size independent from location.

Table 5-1. Summary of sequencing statistics for SSA isolates

| Strain | N50[2] | Total length (bp) | No. of contigs[1] | | | Longest contig (bp) |
|---|---|---|---|---|---|---|
| | | | ≥100bp | ≥ 500bp | ≥1000bp | |
| BF0017 | 138447 | 39725824 | 4455 | 2034 | 1415 | 1043654 |
| BF0032 | 120312 | 40189893 | 5341 | 2406 | 1611 | 701123 |
| BF0048 | 144015 | 40015456 | 4592 | 2099 | 1477 | 742596 |
| BF0005 | 122962 | 40969151 | 6185 | 2299 | 1496 | 661385 |
| BN00293 | 124287 | 40010648 | 6335 | 2373 | 1541 | 980883 |
| JUM1 | 127476 | 40503273 | 4992 | 2252 | 1571 | 597604 |
| NG0104 | 128841 | 38946046 | 4717 | 1965 | 1351 | 659430 |
| NG0110 | 115527 | 39600383 | 5080 | 2254 | 1577 | 880941 |
| NG0135 | 109842 | 39852204 | 5338 | 2339 | 1593 | 538274 |
| NG0153 | 130820 | 39800748 | 5339 | 2209 | 1508 | 743903 |
| TG0004 | 125683 | 39965636 | 5349 | 2448 | 1685 | 907211 |
| TZ0090 | 127695 | 38952040 | 3923 | 1816 | 1322 | 675452 |
| UG0008 | 122477 | 39183782 | 5090 | 2013 | 1392 | 1076399 |
| EG0308 | 149180 | 41564590 | 5528 | 2044 | 1428 | 756898 |
| KE0002 | 147435 | 40252061 | 5101 | 2153 | 1432 | 694455 |
| KE0016 | 156802 | 40270040 | 4818 | 2085 | 1366 | 868332 |
| KE0017 | 141652 | 41104100 | 9348 | 2143 | 1446 | 713476 |
| KE0019 | 176600 | 39371043 | 3334 | 1784 | 1296 | 948236 |
| KE0021 | 152792 | 40076482 | 4437 | 2041 | 1375 | 1032103 |
| KE0029 | 154544 | 41039609 | 7752 | 2251 | 1394 | 677851 |
| KE0210 | 15865 | 38854174 | 9941 | 5637 | 4335 | 99136 |
| KE0255 | 119152 | 39832145 | 5886 | 2271 | 1547 | 531872 |
| KE0041 | 146883 | 39008374 | 3793 | 1858 | 1359 | 908749 |
| KE0332 | 77399 | 41002515 | 5329 | 2425 | 1788 | 460665 |
| KE0415 | 86941 | 39452515 | 2513 | 1596 | 1419 | 579877 |
| KE0443 | 78053 | 40885170 | 5303 | 2292 | 1742 | 495492 |
| KE0473 | 71202 | 40617765 | 5123 | 2284 | 1739 | 373705 |
| KE0491 | 79518 | 40053702 | 2182 | 1676 | 1487 | 454784 |

[1]Contigs generated by *denovo* assembly of raw sequence reads from Illumina paired end sequencing.
[2]Contig length for which 50% of the entire assembly is contained in contigs equal to or larger than this value.

Table 5-2. List of isolates used in this study

| Isolate | Location[1] | Host[2] | Clade[3] | Reference |
|---|---|---|---|---|
| VO104 | Puerto Rico | | Clade 1 | D.M. Soanes unpublished data |
| KE0491 | Lunga Lunga, Kwale county, coastal Kenya | *O. sativa* | Clade 1 | This study |
| TH3 | Thailand | *O. sativa* | Clade 1 | D.M. Soanes unpublished data |
| TH12 | Thailand | *O. sativa* | Clade 1 | Chiapello *et al.* 2015 |
| TH16 | Thailand | *O. sativa* | Clade 1 | Chiapello *et al.* 2015 |
| Guy11 | French Guiana | *O. sativa* | Clade 1 | D.M. Soanes unpublished data |
| HN19311 | Hunan Province, China | *O. sativa* | Clade 1 | Chen *et al*. 2013 |
| 76.3 | China | *O. sativa* | Clade 1 | D.M. Soanes unpublished data |
| TGO004 | Togo | *O. sativa* | | This study |
| KE0021 | Mwea, Kirinyaga county, central Kenya | *O. sativa* | Clade 2 | This study |
| KE0019 | Mwea, Kirinyaga county, central Kenya | *O. sativa* | Clade 2 | This study |
| KE0016 | Mwea, Kirinyaga county, central Kenya | *O. sativa* | Clade 2 | This study |
| KE0017 | Mwea, Kirinyaga county, central Kenya | *O. sativa* | Clade 2 | This study |
| KE0041 | Mwea, Kirinyaga county, central Kenya | *O. sativa* | Clade 2 | This study |

| | | | | |
|---|---|---|---|---|
| KE0002 | Mwea, Kirinyaga county, central Kenya | *O. sativa* | Clade 2 | This study |
| KE0029 | Mwea, Kirinyaga county, central Kenya | *O. sativa* | Clade 2 | This study |
| V0113 | Texas, USA | *O. sativa* | Clade 2 | D.M. Soanes unpublished data |
| VO108 | Louisiana, U.SA | *O. sativa* | Clade 2 | D.M. Soanes unpublished data |
| MG01 | India | *O. sativa* | Clade 2 | Gowda *et al*. 2015 |
| KE0443 | Lunga Lunga, Kwale county, coastal Kenya | *O. sativa* | Clade 2 | This study |
| KE0210 | Ahero, Kisumu county, western Kenya | *O. sativa* | Clade 2 | This study |
| KE0255 | Ahero, Kisumu county, western Kenya | *O. sativa* | Clade 2 | This study |
| KE0332 | Homa-Bay county, western Kenya | *O. sativa* | Clade 2 | This study |
| TN0090 | Tanzania | *O. sativa* | Clade 2 | This study |
| KE0473 | Lunga Lunga, Kwale county, coastal Kenya | *O. sativa* | Clade 2 | This study |
| B157 | India | *O. sativa* | Clade 2 | Gowda *et al*. 2015 |
| KE0415 | Lunga Lunga, Kwale county, coastal Kenya | *O. sativa* | Clade 3 | This study |
| UG0008 | Uganda | *O. sativa* | Clade 3 | This study |
| NGO0110 | Nigeria | *O. sativa* | Clade 3 | This study |
| NGO0104 | Nigeria | *O. sativa* | Clade 3 | This study |

| | | | | |
|---|---|---|---|---|
| BNO0293 | Benin | *O. sativa* | Clade 3 | This study |
| NGO0135 | Nigeria | *O. sativa* | Clade 3 | This study |
| NGO0153 | Nigeria | *O. sativa* | Clade 3 | This study |
| BF0032 | Burkina Faso | *O. sativa* | Clade 3 | This study |
| PH14 | Philippines | *O. sativa* | Clade 3 | Chiapello *et al.* 2015 |
| BF0048 | Burkina Faso | *O. sativa* | Clade 3 | This study |
| BF0017 | Burkina Faso | *O. sativa* | Clade 3 | This study |
| MG10 | India | *O. sativa* | Clade 3 | Shirke *et al.* 2016 |
| KJ201 | Korea | *O. sativa* | Clade 4 | Jeon *et al.* 2015 |
| 82.0535 | China | *O. sativa* | Clade 4 | D.M. Soanes unpublished data |
| Y34 | Yunnan Province, China | *O. sativa* | Clade 4 | D.M. Soanes unpublished data |
| EG308 | Egypt | *O. sativa* | Clade 4 | D.M. Soanes unpublished data |
| FR13 | France | *O. sativa* | Clade 4 | Jeon *et al.* 2015 |
| INA168 | Japan | *O. sativa* | Clade 4 | D.M. Soanes unpublished data |
| P131 | Japan | *O. sativa* | Clade 4 | Xue *et al*. 2012 |
| GLN3 | Hangzhou, China | *O. sativa* | Clade 4 | D.M. Soanes unpublished data |

| | | | | |
|---|---|---|---|---|
| GLN4 | Hangzhou, China | *O. sativa* | Clade 4 | D.M. Soanes unpublished data |
| FJ81278 | Fujian Province, China | *O. sativa* | Clade 4 | Chen *et al*. 2013 |
| 98-08 | Southeast China | *O. sativa* | Clade 4 | Dong *et al*. 2015 |
| 90.4.1 | China | *O. sativa* | Clade 4 | D.M. Soanes unpublished data |
| JUM1 | USA | *O. sativa* | Clade 4 | This study |
| 87 120 2 | China | *O. sativa* | | D.M. Soanes unpublished data |
| BF005 | | *O. sativa* | | This study |
| US71 | USA | *Setaria italica* | | Chiapello *et al.* 2015 |
| BTTrp6 | Bangladesh | Torpedo grass (*Panicum rapens*) | | D.M. Soanes unpublished data |
| BTTrp7 | Bangladesh | Torpedo grass (*P. repens*) | | D.M. Soanes unpublished data |
| BR62 | Brazil | *Eleusine indica* (Indian goosegrass) | | D.M. Soanes unpublished data |

[1] Locality from which the isolate was collected.
[2] Host plant from which the isolate was collected.
[3]Clade obtained by phylogenetic analysis based on Single Nucleotide Polymorphosm as shown in Fig. 5-2.

### 5.4.1 Phylogenetic analysis of SSA *M. oryzae* isolates based on SNP's

We analysed SNPs in *M. oryzae* isolates from rice growing countries in SSA including Kenya, Uganda, Tanzania, Nigeria, Burkina Faso, Togo, Benin and selected reference strains available in the public databases (Table 5-2). These were used to construct a tree to show phylogenetic relationship between these strains, based on a genome-wide analysis. Our analyses separated the isolates into 4 distinct clades, separated by high bootstrap values (100%) for all the clades. With the exception of a few isolates majority of the the SSA isolates separated into 2 major clades with West African isolates clustering distinctly (clade 3) from East African isolates (Clade 2). The Kenyan isolates separated into two sister clades with isolates from Central Kenya (sub-clade 2), distinct to Coastal and Western Kenya isolates (sub-clade1). Isolates collected from non-rice hosts including *Setaria italica, Pennisetum repens* and *Eleusine indica* (singletons A, B and C respectively) clustered as singletons and separately from rice-infecting isolates (Figure 5-2). However, 2 rice-infecting isolates, BF5 and 87.120.2 (singletons E and D respectively) clustered separately from other rice-infecting isolates.

The Kenyan and other SSA isolates clustered together with isolates from India, Philippines, USA and China (Figure 5-2). The majority of Kenyan isolates clustered together with isolates from India, USA and Tanzania.

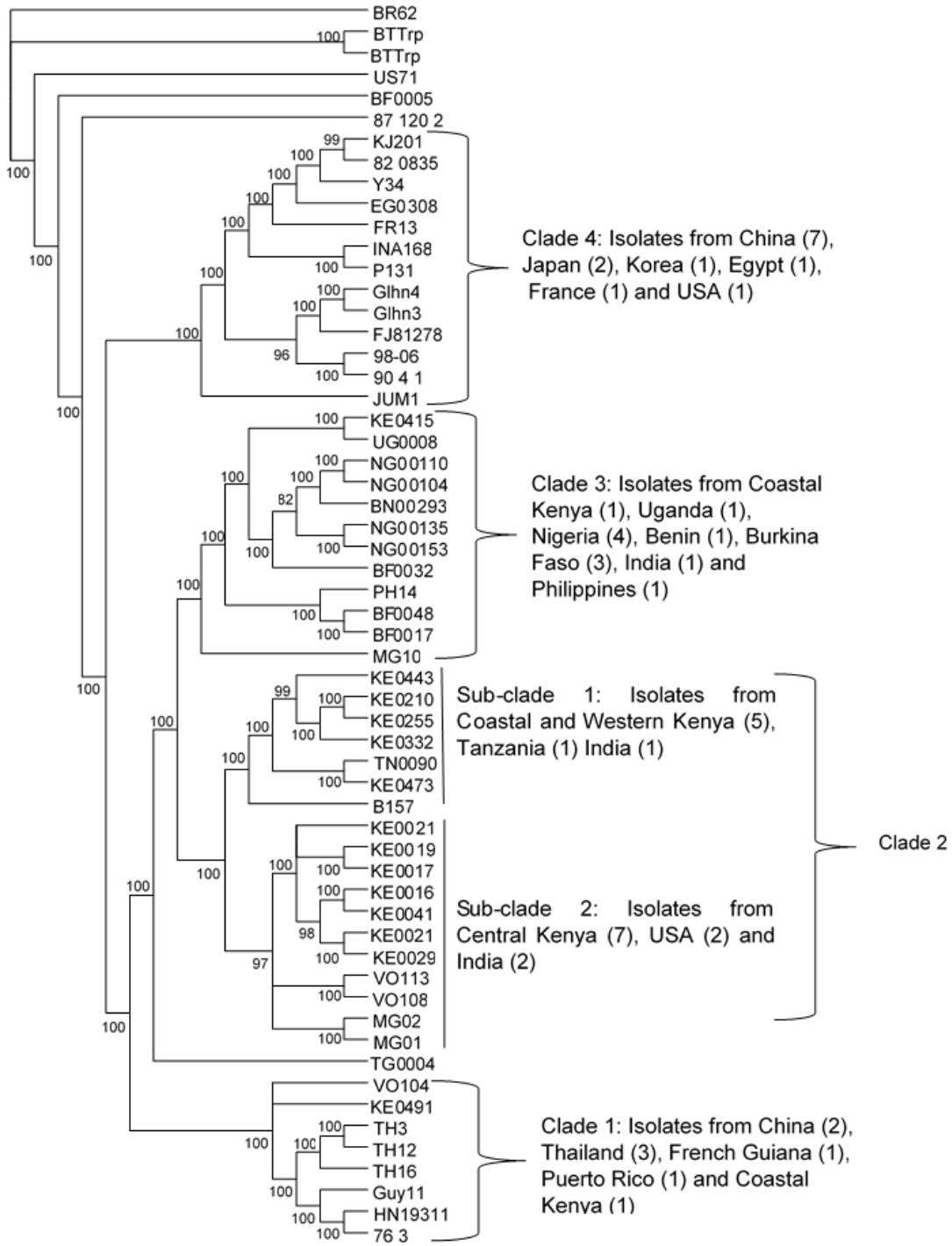


Figure 5-2. Phylogenetic analysis based on Single Nucleotide Polymorphisms (SNPs) for *M. oryzae* isolates from sub-Saharan Africa and reference isolates from other rice growing regions. Detailed information on the isolates is presented in Table 5-2. US71, BTTrp and BR62 were collected from non-rice host plants; BF0005 and 87 120 2 are isolates from Burkina Faso and China respectively collected from rice. Numbers by nodes indicate bootsrap values.

### 5.4.2 A comparison of isolate specific genes between 70-15 and sequenced isolates

Cluster analysis of genome sequences from SSA isolates was performed, as described in section 2.6 and isolate-specific genes were identified. Among the SSA isolates, most isolate specific genes were found in western African isolates with increasing order BF0017< NGO0135< BN00293< NGO0110< BF0005 for 5 isolates with most strain specific genes. Among the East African isolates, KE0332, KE0491 and KE0443 had the highest number of strain specific genes (Table 5-3). A total of 44 genes were conserved in SSA isolates and were absent in Guy 11. Out of these, 19 genes are already annotated (Table 5-4). These genes encode enzymes involved in various functions including the DNA repair pathway (nucleotidyl transferase), DNA methylation (e.g. methyltransferase, histone methyltransferasesC), metabolism of aldehydes, fats (e.g. aldehyde dehydrogenase, lipases), oxidation of organic substrates (e.g. 2OG-Fe(II) oxygenase) and stress signalling (e.g. stress-activated map kinase-interacting protein).

Table 5-3. A comparison of isolate specific genes between 70-15 and sequenced isolates

| Isolate | Both genomes[1] | 70-15 specific | Isolate specific |
|---|---|---|---|
| HN19311 | 11136 | 1661 | 1050 |
| INA168 | 11245 | 1553 | 1433 |
| JUM1 | 11028 | 1760 | 1847 |
| KE0002 | 11194 | 1581 | 1626 |
| KE0016 | 11205 | 1572 | 1651 |
| KE0017 | 11205 | 1575 | 1568 |
| KE0019 | 11229 | 1545 | 1497 |
| KE0021 | 11219 | 1560 | 1620 |
| KE0029 | 11221 | 1563 | 1616 |
| KE0041 | 11201 | 1595 | 1468 |
| KE0332 | 11246 | 1533 | 1676 |
| KE0415 | 11288 | 1493 | 1522 |

| | | | |
|---|---|---|---|
| KE0443 | 11258 | 1518 | 1628 |
| KE0473 | 11247 | 1530 | 1584 |
| KE0491 | 11265 | 1516 | 1652 |
| TZ0090 | 11185 | 1607 | 1516 |
| UG0008 | 11192 | 1595 | 1430 |
| BF0017 | 11083 | 1708 | 1753 |
| BF0032 | 11185 | 1602 | 1649 |
| BF0048 | 11148 | 1641 | 1701 |
| BF0005 | 10940 | 1851 | 1946 |
| BN00293 | 10982 | 1806 | 1829 |
| NG00104 | 11121 | 1673 | 1565 |
| NG00110 | 10955 | 1846 | 1871 |
| NG00135 | 10979 | 1818 | 1793 |
| NG00153 | 11087 | 1706 | 1742 |
| Guy11 | 11458 | 1300 | 1518 |
| EG308 | 11263 | 1524 | 1790 |
| MG02 | 11136 | 1674 | 2044 |
| MG10 | 11127 | 1676 | 1694 |
| P131 | 11216 | 1585 | 1471 |
| TH16 | 11172 | 1629 | 1270 |
| B157 | 11012 | 1784 | 1753 |
| VO104 | 11181 | 1604 | 1704 |
| VO108 | 11183 | 1616 | 1586 |
| VO113 | 11177 | 1615 | 1586 |
| Y34 | 11214 | 1586 | 1523 |
| 76.3 | 11403 | 1381 | 1185 |
| 82.0835 | 11230 | 1561 | 1538 |
| 87.120.2 | 11074 | 1716 | 1493 |
| 90.4.1 | 11257 | 1536 | 1409 |
| 98-06 | 11325 | 1452 | 1668 |
| FJ81278 | 11149 | 1657 | 1331 |
| Glhn3 | 11214 | 1582 | 1496 |
| Glhn4 | 11234 | 1561 | 1488 |

[1]Predicted genes from each genome were clustered with those from 70-15 using proteinortho. Columns show numbers of clusters containing genes from both genomes, 70-15 only and sequenced isolate only.

Table 5-4. List of annotated genes conserved in SSA isolates and absent in Guy

| 70-15 | Broad Institute annotation | BLAST2GO annotation | Pfam |
|---|---|---|---|
| MGG_02390T0 | Hypothetical protein (534 aa) | Translation initiation factor eif-2b subunit gamma | Nucleotidyl transferase (PF00483), Bacterial transferase hexapeptide (PF00132) |
| MGG_11012T0 | Hypothetical protein (190 aa) | | |
| MGG_14658T0 | Hypothetical protein (423 aa) | Tam domain methyltransferase | Methyltransferase domain (PF13489) |
| MGG_01348T0 | Hypothetical protein (863 aa) | Vacuolar fusion protein ccz1 like protein | Fungal domain of unknown function (DUF1712) (PF08217) |
| MGG_12955T0 | Hypothetical protein (825 aa) | Stress-activated map kinase-interacting protein | Stress-activated map kinase interacting protein 1 (SIN1) (PF05422) |
| MGG_05008T0 | Aldehyde dehydrogenase (551 aa) | Aldehyde dehydrogenase | Aldehyde dehydrogenase family (PF00171) |
| MGG_14057T0 | Lipase 5 (381 aa) | Lipase 1 precursor | Secretory lipase (PF03583) |
| MGG_01026T0 | Hydroxymethylglutaryl-CoA synthase (457 aa) | Hydroxymethylglutaryl- synthase | Hydroxymethylglutaryl-coenzyme A synthase N terminal |
| MGG_17527T0 | Hypothetical protein (75 aa) | Hypothetical protein MGG_17527 | |
| MGG_05675T0 | Hypothetical protein (839 aa) | Conserved glutamic acid-rich protein | |
| MGG_07023T0 | Hypothetical protein (316 aa) | Pal1-like protein | |
| MGG_14664T0 | Hypothetical protein (311 aa) | 2og-fe oxygenase superfamily protein | 2OG-Fe(II) oxygenase superfamily (PF13532) |
| MGG_01323T0 | Hypothetical protein (568 aa) | Hypothetical protein MGG_01323 | |
| MGG_04827T0 | Vacuolar ATP synthase subunit H (476 aa) | Vacuolar atp synthase subunit h | V-ATPase subunit H |
| MGG_17998T0 | Hypothetical protein (155 aa) | Hypothetical protein MGG_17998 | |
| MGG_04866T0 | regucalcin (325 aa) | Smp-30 gluconolaconase lre domain-containing protein | SMP-30/Gluconolaconase/LRE-like region (PF08450) |

| | | | |
|---|---|---|---|
| MGG_02749T0 | Hypothetical protein (296 aa) | l-ascorbic acid binding protein | 2OG-Fe(II) oxygenase superfamily (PF13640) |
| MGG_14610T0 | *M. oryzae* 70-15 hypothetical protein (420 aa) | histone-lysine n-methyltransferase | SET domain (PF00856) , Rubisco LSMT substrate-binding (PF09273) , MYND finger01753) |
| MGG_01461T0 | *M. oryzae* 70-15 hypothetical protein (286 aa) | atp synthase f0 | Fungal protein of unknown function (DUF1774) (PF08611) |

### 5.4.3 Distribution of known avirulence genes (*AVR*) in Kenyan *M. oryzae* isolates

With the exception of *PWL3*, *PWL4*, *AVR-CO39* and *AVR-PII* all other known AVRs were present in *M. oryzae* isolates from Kenya. *AVR-PI9, AVR-PIZ-T* and *AVR-PI54* were present in all Kenyan isolates. Differences in distribution of *AVR-PITA* and *AVR-PIA* was observed between central and western Kenya isolates. *AVR-PITA* was present in western Kenya isolates and absent in central Kenya isolates. *AVR-PIA* was present in Central Kenya isolates and absent in Western Kenya isolates (Table 5-5).

With exception of *PWL1*, *PWL3* and *AVR-CO39*, there were no major differences in distribution of known *AVRs* between the West African and East African isolates (Figure 5-3). *AVR-CO39* was identified only in two isolates from Burkina Faso (BF5 and BF17). *PWL1* was present in only East African isolates while *PWL3* was present in only West African isolates. *AVR-PI54*, *AVR-PI9, AVR-PIB,* *AVR-PIZ-T* and *PWL2* were present at relatively higher frequency across Eastern and Western Africa isolates (Figure 5-3).

Table 5-5. Distribution of avirulence genes in Kenyan *M. oryzae* isolates

| Avirulence genes | Central Kenya isolates isolates | | | | | | | Coastal and Western Kenya | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | KE0002 | KE0016 | KE0017 | KE0019 | KE0021 | KE0041 | KE0029 | KE0210 | KE0255 | KE0332 | KE0443 | KE0415 | KE0473 | KE0491 | Guy 11 |
| **PWL1* | A | A | A | A | A | A | A | P | P | P | P | A | P | P | A |
| **PWL2* | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| **PWL3* | A | A | A | A | A | A | A | A | A | A | A | A | A | A | P |
| **PWL4* | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A |
| ***CO39* | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A |
| *AVR PITA* | A | A | A | A | A | A | A | P | P | P | P | A | P | P | P |
| *AVR PIA* | P | P | P | P | P | P | P | A | A | A | A | A | A | A | A |
| *AVR PIK* | A | A | A | A | A | A | A | P | P | P | P | P | P | P | P |
| *AVR PII* | A | A | A | A | A | A | A | A | A | A | A | A | A | A | A |
| *AVR PIZT* | P | P | P | P | P | P | P | P | P | P | P | P | P | A | P |
| *AVR PI9* | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| *AVR PIB* | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |
| **AVR PI54* | P | P | P | P | P | P | P | P | P | P | P | P | P | P | P |

P Presence of avirulence gene; A absence of avirulence gene; ▭ indicates resistant reaction on monogenic line carrying the corresponding R gene; ▭ indicates susceptible reaction on monogenic line carrying the corresponding R gene; * Not tested; ** Infections performed on rice variety Co39.

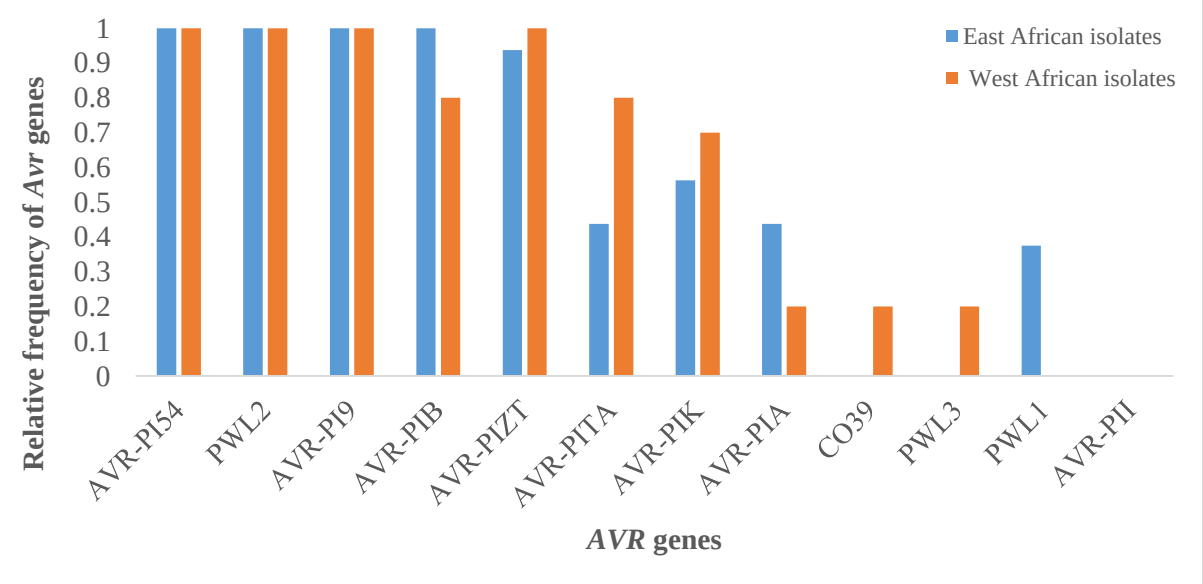


Figure 5-3. Regional distribution of AVR genes in East and West African *M. oryzae* isolates. AVR genes identified by similarity search using BLASTN on genomic sequence of the isolates. East African isolates n= 16 (Kenya 14, Uganda 1 and Tanzania 1); West African isolates n=10 (Burkina Faso 4, Nigeria 4, Togo 1, Benin 1).

## 5.5 Allelic diversity of selected Avr in the Kenyan *M. oryzae* isolates

Whereas the presence or absence of *AVR* genes in some isolates corresponded with the relevant disease reaction on monogenic line harbouring respective R genes, this relationship was not observed for some AVRs. All isolates harbouring *AVR-PIZT* and some isolates harbouring *AVR-PIK*, *AVR-PI9* and *AVR-PIB, AVR-PITA* had compatible reactions on monogenic lines harbouring the corresponding R gene (Table 5-5). We therefore examined allelic diversity of *AVR* genes which were present and showed a compatible reaction on the corresponding R gene.

### 5.5.1 Allelic diversity in *AVR-PITA*

Compared to GenBank *AVR-PITA* sequence (accession no. AF207841.1), sequence nucleotide substitutions and indels were observed in the *AVR-PITA* coding region from KE0491, KE0473, KE0443, and Guy11 (Figure 5-4). No sequence variability was observed between the GenBank *AVR-PITA* sequence and Kenyan isolates KE0225 and KE0332. The sequence variability in the coding region resulted in changes in amino acid sequence (Figure 5-5). A BLAST search of sequences in the *AVR-PITA* promoter region indicated that Inago 1, Inago 2, MGR 608, and MGR 619 solo-LTR sequences were present in GenBank *AVR-PITA* sequence and Kenyan isolates as exemplified in KE0332 and KE0443 (Figure 5-6 – 5-7).

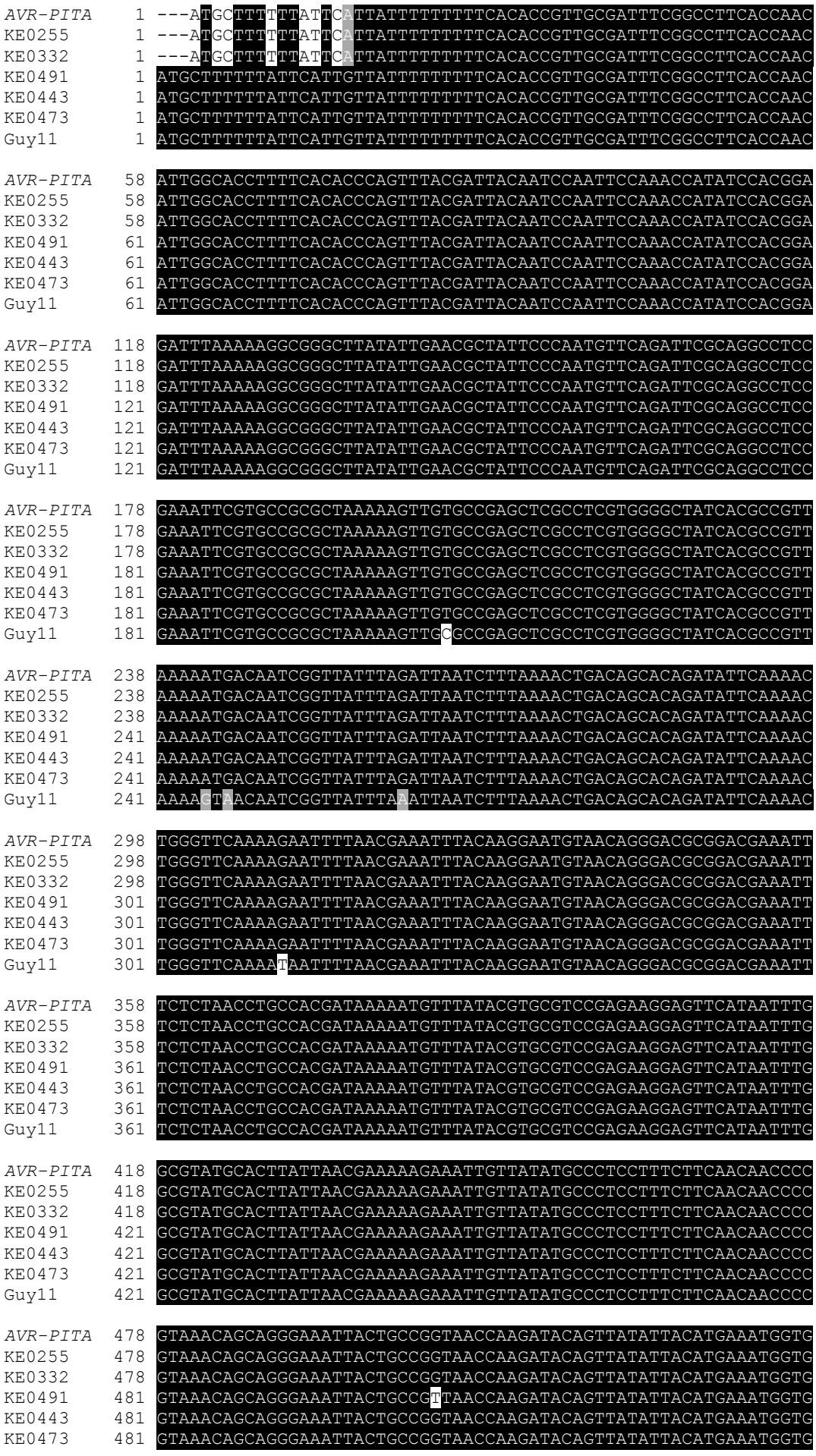
AVR-PITA 1 ---ATGCTTTTTTATTCATTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAAC
KE0255 1 ---ATGCTTTTTTATTCATTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAAC
KE0332 1 ---ATGCTTTTTTATTCATTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAAC
KE0491 1 ATGCTTTTTTATTCATTGTTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAAC
KE0443 1 ATGCTTTTTTATTCATTGTTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAAC
KE0473 1 ATGCTTTTTTATTCATTGTTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAAC
Guy11 1 ATGCTTTTTTATTCATTGTTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAAC
AVR-PITA 58 ATTGGCACCTTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGA
KE0255 58 ATTGGCACCTTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGA
KE0332 58 ATTGGCACCTTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGA
KE0491 61 ATTGGCACCTTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGA
KE0443 61 ATTGGCACCTTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGA
KE0473 61 ATTGGCACCTTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGA
Guy11 61 ATTGGCACCTTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGA
AVR-PITA 118 GATTTAAAAAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCC
KE0255 118 GATTTAAAAAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCC
KE0332 118 GATTTAAAAAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCC
KE0491 121 GATTTAAAAAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCC
KE0443 121 GATTTAAAAAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCC
KE0473 121 GATTTAAAAAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCC
Guy11 121 GATTTAAAAAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCC
AVR-PITA 178 GAAATTCGTGCCGCGCTAAAAAGTTGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTT
KE0255 178 GAAATTCGTGCCGCGCTAAAAAGTTGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTT
KE0332 178 GAAATTCGTGCCGCGCTAAAAAGTTGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTT
KE0491 181 GAAATTCGTGCCGCGCTAAAAAGTTGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTT
KE0443 181 GAAATTCGTGCCGCGCTAAAAAGTTGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTT
KE0473 181 GAAATTCGTGCCGCGCTAAAAAGTTGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTT
Guy11 181 GAAATTCGTGCCGCGCTAAAAAGTTGCGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTT
AVR-PITA 238 AAAAATGACAATCGGTTATTTAGATTAATCTTTAAAACTGACAGCACAGATATTCAAAAC
KE0255 238 AAAAATGACAATCGGTTATTTAGATTAATCTTTAAAACTGACAGCACAGATATTCAAAAC
KE0332 238 AAAAATGACAATCGGTTATTTAGATTAATCTTTAAAACTGACAGCACAGATATTCAAAAC
KE0491 241 AAAAATGACAATCGGTTATTTAGATTAATCTTTAAAACTGACAGCACAGATATTCAAAAC
KE0443 241 AAAAATGACAATCGGTTATTTAGATTAATCTTTAAAACTGACAGCACAGATATTCAAAAC
KE0473 241 AAAAATGACAATCGGTTATTTAGATTAATCTTTAAAACTGACAGCACAGATATTCAAAAC
Guy11 241 AAAAGTAACAATCGGTTATTTAAATTAATCTTTAAAACTGACAGCACAGATATTCAAAAC
AVR-PITA 298 TGGGTTCAAAAGAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATT
KE0255 298 TGGGTTCAAAAGAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATT
KE0332 298 TGGGTTCAAAAGAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATT
KE0491 301 TGGGTTCAAAAGAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATT
KE0443 301 TGGGTTCAAAAGAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATT
KE0473 301 TGGGTTCAAAAGAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATT
Guy11 301 TGGGTTCAAAATAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATT
AVR-PITA 358 TCTCTAACCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTG
KE0255 358 TCTCTAACCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTG
KE0332 358 TCTCTAACCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTG
KE0491 361 TCTCTAACCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTG
KE0443 361 TCTCTAACCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTG
KE0473 361 TCTCTAACCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTG
Guy11 361 TCTCTAACCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTG
AVR-PITA 418 GCGTATGCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCC
KE0255 418 GCGTATGCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCC
KE0332 418 GCGTATGCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCC
KE0491 421 GCGTATGCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCC
KE0443 421 GCGTATGCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCC
KE0473 421 GCGTATGCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCC
Guy11 421 GCGTATGCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCC
AVR-PITA 478 GTAAACAGCAGGGAAATTACTGCCGGTAACCAAGATACAGTTATATTACATGAAATGGTG
KE0255 478 GTAAACAGCAGGGAAATTACTGCCGGTAACCAAGATACAGTTATATTACATGAAATGGTG
KE0332 478 GTAAACAGCAGGGAAATTACTGCCGGTAACCAAGATACAGTTATATTACATGAAATGGTG
KE0491 481 GTAAACAGCAGGGAAATTACTGCCGTTAACCAAGATACAGTTATATTACATGAAATGGTG
KE0443 481 GTAAACAGCAGGGAAATTACTGCCGGTAACCAAGATACAGTTATATTACATGAAATGGTG
KE0473 481 GTAAACAGCAGGGAAATTACTGCCGGTAACCAAGATACAGTTATATTACATGAAATGGTG


Figure 5-4. Multiple sequence alignment of *AVR-PITA* coding sequences for selected Kenyan *M. oryzae* isolates, GenBank AVR-PITA sequence accession no. AF207841.1 and Guy 11. KE0255, KE0332, KE0491, KE0443 and Guy11 are compatible with monogenic line carrying Pita R gene.

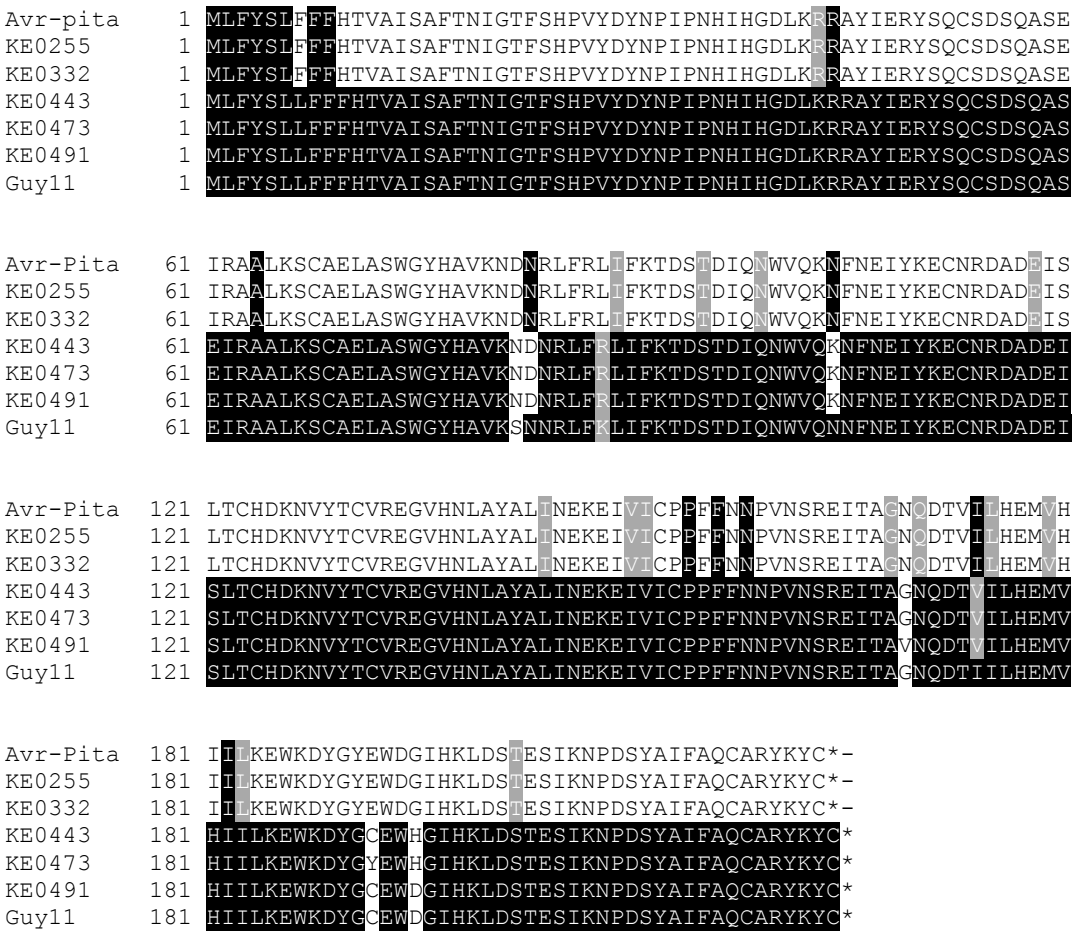


Figure 5-5. Multiple amino acid sequence alignment of Avr-Pita in Kenyan *M. oryzae* isolates, reference GenBank AVR-PITA sequence accession no. AF207841.1 and Guy11. KE0255, KE0332, KE0491, KE0443 and Guy11 are compatible with monogenic line carrying Pita R gene.

TAGACTAGCTTCCGTGCTATGTTTACCCTGGCCGTGACAACTACCATGGAACCCAAGAT
TGTTAGAGGACATTGTAAACCTGACGATAATCTCTGCACGCCGAATATATGCGACAATT
TAAAGGCATATTAAAATATAGCCAACCGCCAAATAAATTCCGTACTAACTAAGCATATT
TTCAAAAGGGGTTCGGAAACTGCACTGTGGCTACATTGTAGGTAAAACGGGCAAATATT
GTTCAGCTTAGGTATTTGCTTAGATTTGACGGAATTCCATACCTGCCTAATTTTGACCA
CAAATTAGAGAACGTAATCCGAACCAAGCTTTTAGTGTTGCCAACGTGATACGGAGTTT
TTGCTGCCGAGTCTGCCGGGCAAAAACGGAACCCAATGTCACGGCCAGGCATACATTGG
AGAGCCTCAGTGTATTAGGCGCTATTAACGAAAATTCTAAACTGAAGAGAAGAGAGAAA
TTACAATCGACGACGCGCTCAAGAGACGCGCTTGAATCCGGAGTTAGTGGACCCTTGTC
CGATCCCTGGCTCGGCGTGGAGCCGAGTCGTTCTGAGGGTAGGTCTAGGGGCCTGATCC
TCACAATATTTTTGTAAATTTCAAAAGTCAGGGAGCATGAATTATGTAGTTATTAATAA
TATGGGCCCAACTCTTACCTTATATAAAATTGTGGATGATATACTAATAAAAGTGGACC
TAATTACCTGCATAATAATGCAGATAATTAACACTAGCAAAATATAATTCGATAATATT
ATTAATGCTAAATAACGCATTAATAAACCAAATAAGTTTTACATCTTCCTAAAGCTTTG
AAAAAAGTCAAGCTGAAATAATAAATAAGTTGGCGTTGTTATAAAATCGACCCGTTTCC
GCCTTTATTGGTTTAATTCGGATAGAGAACATTTTGCTTATAATTCCAAACATACAAAC
AATTATCCACTGACTGAAAATCGACAGTTTTGTTTGCACAATCAACATTATAATTACAA
TTAAAAACTTCTGCACAATTAACATTATTTTTGCAATTATGCTTTTTTATTCATTATTT
TTTTTTCACACCGTTGCGATTTCGGCCTTCACCAACATTGGCACCTTTTCACACCCAGT

TTACGATTACAATCCAATTCCAAACCATATCCACGGAGATTTAAAAAGGCGGGCTTATA
TTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCCGAAATTCGTGCCGCGCTAAAA
AGGTAAATTGAAACTCTTTAAAACAAATTCGGAAAACAATGTTAAATATTTTGTTTAGT
TGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTTAAAAATGACAATCGGTTATTTAG
ATTAATCTTTAAAACTGACAGCACAGATATTCAAAACTGGGTTCAAAAGAATTTTAACG
AAATTTACAAGGAATGTAACAGGGACGCGGACGAAATTTCTCTAACCTGCCACGATAAA
AATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTGGCGTATGCACTTATTAACGA
AAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCCGTAAACAGCAGGGAAATTA
CTGCCGGTAACCAAGATACAGTTATATTACATGAAATGGTGCATATAATTTTAAGTAAG
TTTGCTTTTACAAATTGATAAAACATTTACAAAAGTTTATTTATAAAAATTTTCAAAAA
CTAAAAATTCAAATTTTTATTTAGAAGAGTGGAAAGATTATGGTTACGAATGGGATGGG
ATTCACAAGTAAGTTGTCGAAAAACAAATTTGCTGAATGTTGTTTTATGTTGATAAATT
CTAATTAATATTAAGATTGGATAGTACAGAAAGTATTAAAAACCCCGACAGTTATGCTA
TTTTTGCACAATGTGCACGTTATAAATATTGTTAA

Figure 5-6. AVR-PITA coding sequences and promoter region in KE0332 showing presence of solo-LTR sequences of MGR 608 and MGR 619 elements in the promoter region upstream the coding region. AVR-PITA coding sequences are shown in red.

GTCACGGCCAGGCATACATTGGAGAACCTCAGTGTATTAGGCGCTATCGACGAAAATTC
TAAACTGAAGAGAAGAGAGAAATCACGATTGACGGCGCGCTGGAGAGACGCGCTTAAAT
CCGAGGTCAGTGGATCCCTTTGTCCGATCCCTGGCTCAGCGTGGCTGGTAGAGCCGAGT
CGTGATTCTGAGGGTAGGTCTGGGGGCCTGATCCTCACACCTGGTGGGGCTTTTTAATT
TCCGTTTAAATGTAGGGTTACATTAAAATACCTCCCCAATGTGACATTTGGACCATGAG
ATCACCAGACCCTAACCCTGACCCTGGACCAACGACCCACCAGGGTGATACCTTTATCG
ACGTCGCCTCAAGCTCAGAACTTTGTTTGTTTCCTTTCCTCTCCTCAGAGTAGAATTTT
CGTCGATAGATGCCAATAGACTAGCTTCCGTGCTATGCTTACCCCGGGCCGTGACACTA
ACTAAGCATATTTTCAAAAGGGGTTCGGAAACTGCACTGTGGCTACATTGTAGGTAAAA
CGGGCAAATATTGTTCAGCTTAGGTATTTGCTTAGATTTGACGGAATTCCATACCTGCC
TAATTTTGACCACAAATTAGAGAACGTAATCCGAACCAAGCTTTTAGTGTTGCCAACGT
GATACGGAGTTTTTGCTGCCGAGTCTGCCGGCAAAAACGGAACCCAATGTCACGGCCAG
GCATACATTGGAGAGCCTCAGTGTATTAGGCGCTATTAACGAAAATTCTAAACTGAAGA
GAAGAGAGAAATTACAATCGACGACGCGCTCAAGAGACGCGCTTGAATCCGGAGTTAGT
GGACCCTTGTCCGATCCCTGGCTCGGCCTGGAGCCGAGTCGTTCTGAGGGTAGGTCTAG
GGGCCTGATCCTCACAATATTTTTGTAAATTTCAAAAGTCAGGGAGCATGAATTATGTA
GTTATTAATAATATGGGCCCAACTCTTACCTTATATAAAATTGTGGATGATATACTAAT
AAAAGTGGACCTAATTACCTGCATAATAATGCAGATAATTAACACTAGCAAAATATAAT
TCGATAATATTATTAATGCTAAATAACGCATTAATAAACTAAATAAGTTTTACATCTTC
CTAAAGCTTTGAAAAAAGTCAAGCTGAAATAATAAATAAGTTGGCGTTGTTATAAAATC
GACCCGTTTCCGCCTTTATTGGTTTAATTCGGATAGAGAACATTTTGCTTATAATTCCA
AACATACAAACAATTATCCACTGACTGAAAATCGACAGTTTTGTTTGCACAATCAACAT
TATAATTACAATTAAAAACTTCTGCACAATTAACATTATTTTTGCAATTATGCTTTTTT
ATTCATTGTTATTTTTTTTTCACACCGTTGCGATTTCGGCCTTCACCAACATTGGCACC
TTTTCACACCCAGTTTACGATTACAATCCAATTCCAAACCATATCCACGGAGATTTAAA
AAGGCGGGCTTATATTGAACGCTATTCCCAATGTTCAGATTCGCAGGCCTCCGAAATTC
GTGCCGCGCTAAAAAGGTAAATTGAAACTCTTTAAAACAAATTCGGAAAACAATGTTAA
ATATTTTGTTTAGTTGTGCCGAGCTCGCCTCGTGGGGCTATCACGCCGTTAAAAATGAC
AATCGGTTATTTAGATTAATCTTTAAAACTGACAGCACAGATATTCAAAACTGGGTTCA
AAAGAATTTTAACGAAATTTACAAGGAATGTAACAGGGACGCGGACGAAATTTCTCTAA

CCTGCCACGATAAAAATGTTTATACGTGCGTCCGAGAAGGAGTTCATAATTTGGCGTAT
GCACTTATTAACGAAAAAGAAATTGTTATATGCCCTCCTTTCTTCAACAACCCCGTAAA
CAGCAGGGAAATTACTGCCGGTAACCAAGATACAGTTATATTACATGAAATGGTGCATA
TAATTTTAAGTAAGTTTGCTTTTACAAATTGATAAAACATTTACAAAAGTTTATTTATA
AAAATTTTCAAAAACTAAAAATTCAAATTTTTATTTAGAAGAGTGGAAAGATTATGGTT
GCGAATGGCATGGGATTCACAAGTAAGTTGTCGAAAAACAAATTTGCTGAATGTTGTTT
TATGTTGATAAATTCTAATTAATATTAAGATTGGATAGTACAGAAAGTATTAAAAACCC
CGACAGTTATGCTATTTTTGCACAATGTGCACGTTATAAATATTGTTAA

Figure 5-7. AVR-PITA coding sequences and promoter region in KE0443 showing presence of solo-LTR's sequences of Inago 1 and Inago 2 in the promoter region of AVR-PITA. AVR-PITA coding sequences are shown in red.

### 5.5.2 Allelic diversity in the coding region of *AVR-PIK* in *M. oryzae* isolates from Kenya

Compared to the GenBank *AVR-PIK* sequence, accession no. AB498876.1, Guy11 and the Kenyan *M. oryzae* isolates KE0491, KE0473, KE443, KE0415, and KE0255 showed nucleotide substitution in the *AVR-PIK* coding region (Figure 5-8). No transposon sequences were identified in the promoter region of the isolates. The nucleotide substitutions in the coding regions, resulted in changes in the amino acid sequence (Figure 5-9).

```
AVR-PIK     1 ATGCGTGTTACCACTTTTAACACATTCCTTCTCACTTTGGGAACTGTCGCTGTCGTCAATGCCGAAACGG
KE0491      1 ATGCGTGTTACCACTTTTAACACATTCCTTCTCACTTTGGGAACTGTCGCTGTCGTCAATGCCGAAACGG
KE0473      1 ATGCGTGTTACCACTTTTAACACATTCCTTCTCACTTTGGGAACTGTCGCTGTCGTCAATGCCGAAACGG
Guy11       1 ATGCGTGTTACCACTTTTAACACATTCCTTCTCACTTTGGGAACTGTCGCTGTCGTCAATGCCGAAACGG
KE0443      1 ATGCGTGTTACCACTTTTAACACATTCCTTCTCACTTTGGGAACTGTCGCTGTCGTCAATGCCGAAACGG
KE0255      1 ATGCGTGTTACCACTTTTAACACATTCCTTCTCACTTTGGGAACTGTCGCTGTCGTCAATGCCGAAACGG
KE0415      1 ATGCGTGTTACCACTTTTAACACATTCCTTCTCACTTTGGGAACTGTCGCTGTCGTCAATGCCGAAACGG

AVR-PIK    71 GCAACAAATATATAGAAAAACGCGCTATCGACCTAAGTCGAGAGCGAGACCCTAACTTTTTCGACAACGC
KE0491     71 GCAACAAATATATAGAAAAACGCGCTATCGACCTAAGTCGAGAGCGAGACCCTAACTTTTTCGACAACGC
KE0473     71 GCAACAAATATATAGAAAAACGCGCTATCGACCTAAGTCGAGAGCGAGACCCTAACTTTTTCGACAACCC
Guy11      71 GCAACAAATATATAGAAAAACGCGCTATCGACCTAAGTCGAGAGCGAGACCCTAACTTTTTCGACAACCC
KE0443     71 GCAACAAATATATAGAAAAACGCGCTATCGACCTAAGTCGAGAGCGAGACCCTAACTTTTTCGACCACCC
KE0255     71 GCAACAAATATATAGAAAAACGCGCTATCGACCTAAGTCGAGAGCGAGACCCTAACTTTTTCGACCACCC
KE0415     71 GCAACAAATATATAGAAAAACGCGCTATCGACCTAAGTCGAGAGCGAGACCCTAACTTTTTCGACAACCC

AVR-PIK   141 TGATATTCCTGTACCCGAATGTTTTTGGTTTATGTTTAAAAACAACGTACGTCAAGATGCTGGAACCTGT
KE0491    141 TGATATTCCTGTACCCGAATGTTTTTGGTTTATGTTTAAAAACAACGTACGTCAAGATGCTGGAACCTGT
KE0473    141 TGGTATTCCTGTACCCGAATGTTTTTGGTTTATGTTTAAAAACAACGTACGTCAAGATGATGGAACCTGT
Guy11     141 TGGTATTCCTGTACCCGAATGTTTTTGGTTTATGTTTAAAAACAACGTACGTCAAGATGATGGAACCTGT
KE0443    141 TGGTATTCCTGTACCCGAATGTTTTTGGTTTATGTTTAAAAACAACGTACGTCAAGATGCTGGAACCTGT
KE0255    141 TGGTATTCCTGTACCCGAATGTTTTTGGTTTATGTTTAAAAACAACGTACGTCAAGATGCTGGAACCTGT
KE0415    141 TGGTATTCCTGTACCCGAATGTTTTTGGTTTATGTTTAAAAACAACGTACGTCAAGATGCTGGAACCTGT

AVR-PIK   211 TACAGCTCTTGGAAAATGGACATGAAAGTTGGTCCAAACTGGGTCCATATTAAATCAGACGATAATTGCA
KE0491    211 TACAGCTCTTGGAAAATGGACAAGAAAGTTGGTCCAAACTGGGTCCATATTAAATCAGACGATAATTGCA
KE0473    211 TACAGCTCTTGGAAAATGGACATGAAAGTTGGTCCAAACTGGGTCCATATTAAATCAGACGATAATTGCA
Guy11     211 TACAGCTCTTGGAAAATGGACATGAAAGTTGGTCCAAACTGGGTCCATATTAAATCAGACGATAATTGCA
KE0443    211 TACAGCTCTTGGAAAATGGACATGAAAGTTGGTCCAAACTGGGTCCATATTAAATCAGACGATAATTGCA
KE0255    211 TACAGCTCTTGGAAAATGGACATGAAAGTTGGTCCAAACTGGGTCCATATTAAATCAGACGATAATTGCA
KE0415    211 TACAGCTCTTGGAAAATGGACATGAAAGTTGGTCCAAACTGGGTCCATATTAAATCAGACGATAATTGCA
```


Figure 5-8. Multiple sequence alignment for coding region sequences of AVR-PIK for Kenyan *M. oryzae* isolates. KE0491, KE0473, KE0443, KE0255, KE0415, reference AVR-PIK GenBank sequence accession no. AB498876.1 and Guy11. KE0491, KE0473, KE0443, KE0255, KE0415 and Guy11 are compatible with monogenic line IRBLK-KA harbouring Pik R gene.

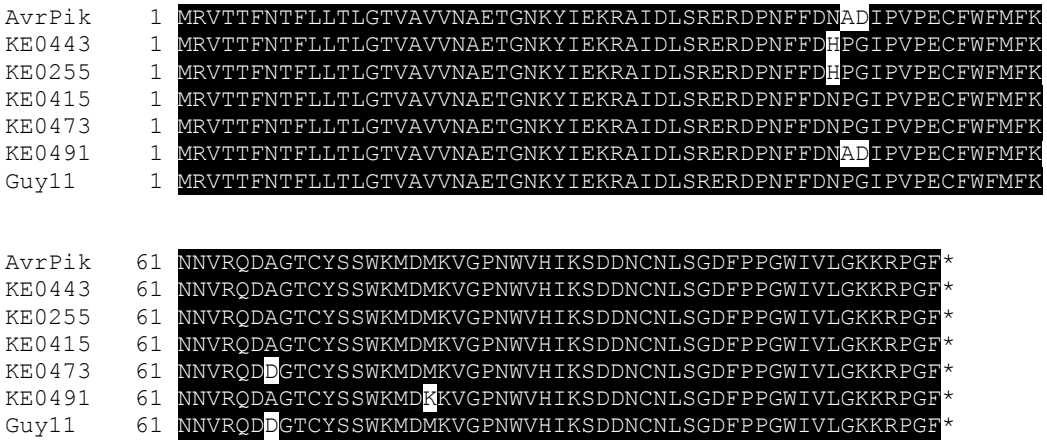


Figure 5-9. Multiple amino acid sequence alignment for Avr-Pik in Kenyan *M. oryzae* isolates. KE0491, KE0473, KE0443, KE0255, KE0415, reference AVR-PIK GenBank sequence accession no. AB498876.1 and Guy11. KE0491, KE0473, KE0443, KE0255, KE0415 and Guy11 are compatible with monogenic line IRBLK-KA harbouring Pik R gene.

### 5.5.3 Allelic diversity in *AVR-PIZ-T* and *AVR-PI9*

There was no sequence variability in the coding region of any of the isolates possessing *AVR-PIZ-T* and *AVR-PI9.*

## 5.6 Identification of a putative effector from Kenyan isolate KE0002

Based on computational identification of effector genes using Effector-P program (Sperschneider *et al.,* 2016), candidate effector genes in KE0002 were provided by Darren Soanes (University of Exeter, School of Biological Sciences). A list of all predicted effectors identified in KE0002, is shown in Table 5-6.

Table 5-6. Predicted effector gene in KE0002 based on Effector-P program

| Gene Name | Length | Cysteine content | BLAST2GO annotation |
|---|---|---|---|
| KE002Y1_contigs.g1117.t1 | 104 | 4 | Hypothetical Y34 |
| KE002Y1_contigs.g9192.t1 | 162 | 6 | Elicitor protein |
| KE0002_13250 | 82 | 6 | Hypothetical MGCH7 |
| KE0002_1434 | 97 | 2 | Hypothetical Y34 |
| KE0002_7508 | 115 | 0 | Hypothetical 7bg7.17 |
| KE0002_4999 | 85 | 12 | Hypothetical Y34 |
| KE0002_5442 | 120 | 0 | Hypothetical Y34 |
| KE0002_6605 | 143 | 3 | Hypothetical Y34 |
| KE0002_6959 | 103 | 8 | Hypothetical Y34 |
| KE0002_8917 | 123 | 5 | Hypothetical Y34 |
| KE0002_10623 | 228 | 0 | Hypothetical 7bg.17 |
| KE0002_10686 | 103 | 0 | Hypothetical 7bg.17 |
| KE0002_14611 | 40 | 1 | Hypothetical Y34 |
| KE0002_15475 | 122 | 2 | Hypothetical Y34 |
| KE0002_16696 | 121 | 4 | Hypothetical Y34 |

Functional characterisation of one of the candidate genes (KE002Y1_contigs.g1117.t1) was undertaken. The presence/absence of the candidate gene in Kenyan isolates and Guy 11 correlated with pathotype data on some monogenic line IRBL Z5-CA harbouring *Piz5* (Table 5-7) and was therefore suspected to be *AVR-PIZ5*. RNA-seq analysis undertaken by Vincent Were (University of Exeter, School of Biological Sciences) indicated that the effector gene is highly expressed during infection of KE0002 on a susceptible variety, Mokoto.

Table 5-7. Correlation between pathotype on IRBLZ5-CA and presence or absence of the candidate gene

| **Response on monogenic line and Candidate AVR** | **Isolates** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GUY 11 | KE0002 | KE0016 | KE0017 | KE0019 | KE0021 | KE0029 | KE0041 | KE0210 |
| Pathotype on IRBLZ5-CA[1] | S | R | R | R | R | R | R | R | R |
| Present/Absence of the candidate gene[2] | A | P | P | P | P | P | P | P | P |

[1] Response of isolate on monogenic line IRBLZ5-CA harbouring *Piz5;* S, susceptible; R resistant

[2] Presence or absence of the gene based on BLAST; A absent; P present.

Guy 11 was complemented with DNA fragment of size 3025 bp comprising the promoter, coding region and terminator sequence of the candidate. Pathotype analysis on monogenic lines harbouring R gene cognate to the targeted *AVR* genes (*AVR* genes that are not yet cloned) showed that Guy 11 was compatible with all the lines. Three Guy11 strains complemented with the candidate effector were compatible to the monogenic lines (Table 5-8) (Figure 5-10).

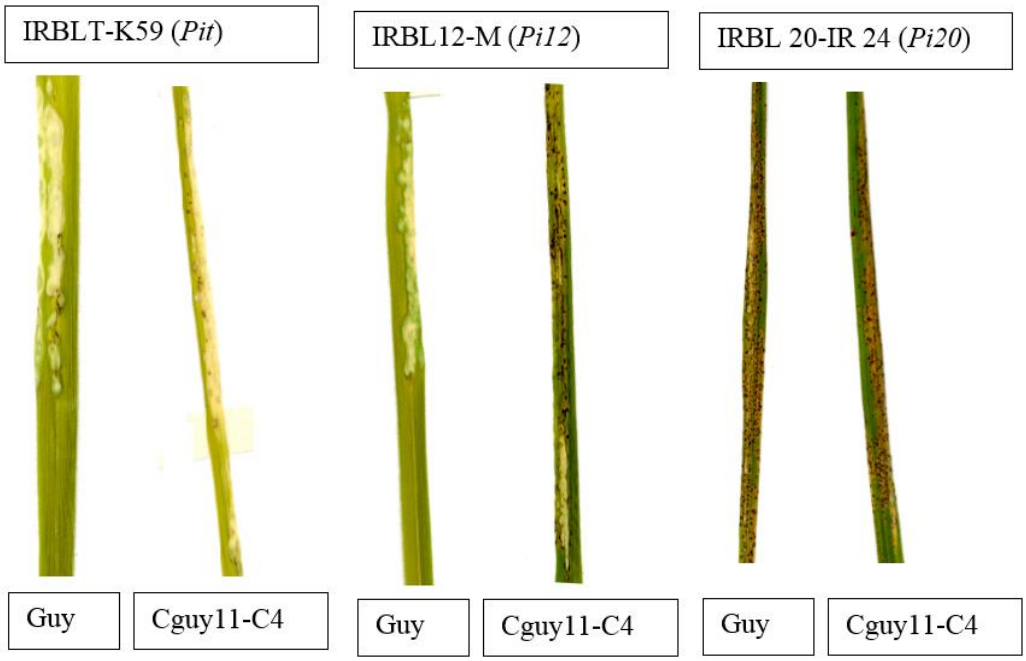


Figure 5-10. Response of Guy11 and strains complemented with putative candidate effector genes on selected monogenic lines

Table 5-8. Pathotype analysis of Guy 11 and strains complemented with putative candidate effector genes

| | | | | **Strains complemented with candidate gene[2]** | | |
|---|---|---|---|---|---|---|
| **Monogenic line[1]** | **R gene** | **KE0002** | **Guy11** | **Cguy11 C4** | **Cguy11 13** | **Cguy11 F** |
| IRBLZ 5-CA (R) | *Piz5* | R[3] | S[4] | S | S | S |
| IRBL 20-IR 24 | *Pi20(t)* | R | S | S | S | S |
| IRBLT-K59 | *Pi-t* | R | S | S | S | S |
| IRBL 12-M | *Pi12(t)* | R | S | S | S | S |
| IRBL 19-A | *Pi19(t)* | R | S | S | S | S |
| IRBLSH-S | *Pish* | R | S | S | S | S |
| IRBL 5-M | *Pi5* | R | S | S | S | S |
| IRBLI-F5 | *Pii* | R | S | S | S | S |
| IRBL 7-M | *Pi7(t)* | R | S | S | S | S |
| IRBLTA 2-RE | *Pita2* | R | S | S | S | S |
| IRBL 3-CP4 | *Pi3* | R | S | S | S | S |
| LTH | | S | S | S | S | S |

[1] Monogenic lines harbouring single R gene in the background of a rice blast susceptible variety, LTH

[2] Guy 11 complemented with candidate effector gene. 3 strains carrying the fragment were used.

[3] Resistant response on the monogenic line.

[4] Susceptible response on the monogenic line.

## 5.7 Discussion

Evolution is a continuous process in which genetic changes over successive generations confer traits to organisms that increase fitness and enable them adapt to a changing environment. Transposable elements are often found in transposable repeat-rich regions, that are enriched with genes that encode effector proteins (McDonald, 1993). The strong association between the transposable elements and genes that encode for effectors indicates that transposable elements may play a crucial role in the evolution of effector genes. Transposons may, for example, contribute to evolution of genomes through several mechanisms. A transposon may be inserted in the coding region or adjacent region, thereby resulting in inactivation of the gene and a new virulence phenotype. In *M. oryzae,* the contribution of transposable elements in evolution *AVR-PITA* and *AVR-PIZ-T* are documented. Insertion of Pot3 element in the coding sequence or in the promoter and sequence variability in the coding sequences have been associated with loss of function of *AVR-PITA* for isolates from USA, India, China, Thailand among other countries. Similarly, insertion of Pot3 element in Guy11 rendered *AVR-PIZ-T* non-functional (Zhou *et al.,* 2007; Li *et al.,* 2012; Singh *et al.,* 2014). Further, loss of function in *AVR-PIZ-T* locus in isolates from a wide geographical distribution has been attributed to sequence variability in the *AVR-PIZ-T* coding region (Chenxi *et al.,* 2014).

*AVR-PITA* gene is located adjacent to a telomere region on chromosome 3 with the region being associated with a large number of transposable elements and high variability. The proportion of repeat sequences in the telomere region has been estimated at 24% compared to an overall genome proportion of 9.7% (Rehmeyer *et al.,* 2006). Our study shows that the promoter region of the *AVR-PITA* of Kenyan isolates is associated with solo-LTRs including Inago1, Inago 2,

MGR608 and MGR619. Solo-LTRs have also been associated with the promoter region of the original *AVR-PITA* gene reported by Orbach *et al.* (2000). During excision of LTRs, some LTR sequences are not completely excised and small segments of LTR sequences are left behind as remnants of the full length LTR transposon. These sequences (referred to as solo-LTRs) are indicative of previous transposon activity in the region. Although the solo-LTRs may not have very apparent effects on function of the gene downstream, it has been suggested they may have subtle effects on virulence and may not be detected by conventional infection assays. According to Orbach *et al.* (2000), future studies aimed at evaluating the role of the solo-LTR on regulation of the downstream avirulence genes may give insights on their role in virulence of *AVR-PITA*. More importantly, the association of *AVR-PITA* with the solo LTRs, particularly Inago 1, has been implicated in the multiple translocation of *AVR-PITA* in rice infecting *M. oryzae*. Compared to other host-specific forms, the *AVR-PITA* chromosomal position was most variable in rice infecting *M. oryzae* and is attributed to periodic deployment of *Pita* resistance gene in rice.

Our study findings indicate that indels were present in the coding regions of *AVR-PITA* and *AVR-PIK* leading to changes in amino acid sequences. Other studies (Zhang *et al.,* 2015; Orbach *et al.,* 2000) have reported nucleotide insertions, deletions and substitutions as some of the strategies employed by *M. oryzae* to evade recognition by the host resistance genes. Interestingly, our results show that all isolates harbouring *AVR-PIZ-T, AVR-PI9* and a few isolates harbouring *AVR-PIB* had lost function towards the cognate R genes. However, there were no nucleotide polymorphisms detected in the coding region and no transposon sequences were identified in the promoter regions of these genes. Isolates harbouring *AVR-PIZ-T* produced large lesions (lesion type 4) and therefore errors

arising from disease assessment are unlikely. Further analysis showed that the cognate R gene (*Piz-t*) was present in the monogenic line. There is need to undertake further studies to understand the interaction between these isolates and the host plant and clarify the loss of function associated with these isolates.

Our study findings indicate that *AVR-PII* was absent in all the African isolates. This could be due to lack of selective pressure imposed by the corresponding R gene either because the cognate R gene is absent from the African germplasm or is not widely used in breeding programmes and therefore there was no necessity for the pathogen to adapt. The converse may also be true. It may be that the cognate R gene is prevalent in African germplasm and therefore the pathogen evolved to avoid host recognition by deleting the *AVR-PII* gene. Previous studies have shown gene loss is a strategy employed by pathogen to evolve and adapt to new environments (Powell *et al.,* 2008).

Our study shows that with the exception of two isolates, from Burkina Faso, BF5 and BF17, *AVR-CO39* was absent in other SSA isolates studied. This is consistent with a study by Tosa *et al.,* (2005) that showed *AVR-CO39* was almost exclusively absent in all rice-infecting isolates but present in other non-rice infecting *M*. *oryza*e species and therefore confers avirulence at host species level. Few rice-infecting isolates contained a non-functional *AVR-CO39* in which half of the 5' end was deleted. The occurrence of the full length *AVR-CO39* copies in BF5 and BF17 may be an indication that the original host may possibly be a non-rice plant, for example *Eleusine* spp., and that the isolates may have colonised rice through opportunistic infections. Unlike in other fungi, adaptation of *M. oryzae* to different host plants is not associated with major modification in gene content or gene family organisation with proportion of different categories of genes involved in pathogenicity being conserved across genomes (Chiapello

*et al.,* 2015). According to the authors, host adaptation is due to occurrence of a small number of lineage-specific genes. In their study the authors identified 529 families specific to non-rice host and 84 gene families specific to rice-host isolates. Host specificity may also be controlled by a few avirulence genes as exemplified by the loss of *AVR-CO39* in rice isolates (Farman *et al.,* 2002).

Comparative genomic analysis of Kenyan isolates indicates that that the rice blast isolates from central Kenya are distinct to those from western and coastal Kenya. Isolates from western and coastal Kenya are closely related and clustered together. These results confirm our DNA fingerprinting results and confirms the robustness of Pot2 element in DNA fingerprinting of *M. oryzae.* The differences in genetic structure between *M. oryzae* isolates from rice growing regions in Kenya may be due to differences in selection pressure imposed by R genes of varieties grown in these regions. As previously explained, rice cultivation in central Kenya is dominated by a single variety (Basmati 370) whereas there is more heterogeneity in rice varieties cultivated in coastal and western regions. Differences in selection pressure imposed by R genes may also have contributed to the genetic differences observed at regional level between East and West African isolates. These two groups clustered separately indicating they are genetically distinct. West Africa has a longer history of rice cultivation than East Africa. The African rice, *O. glaberima*, is thought to have been originated from its wild ancestor *O. barthii* which is known to have been cultivated along the Niger River 2000-3,000 years ago (Linares, 2002). Due to a longer history of rice cultivation in the West Africa, there is a relatively higher diversity of varieties cultivated compared to East Africa and therefore differences in selection pressure imposed by R genes in these two regions. African rice blast isolates clustered with isolates from other regions including India, China, Philippines, USA and

Thailand. For example, Kenyan isolates clustered very closely to Indian isolates (MG01 and MG02) and USA isolates (VO113 and V0108). This indicates that African isolates are closely related to isolates from Asia and America. Rice cultivation has a longer history in South East Asia where it has been cultivated for 8,000-9,000 years compared to 3,000 years in Africa (Linares, 2002; Callaway 2014). Although there are conflicting reports on when Asian rice was first introduced in Africa, it has been proposed that in the 16$^{th}$ Century, the Portuguese played a critical role in introduction of Asian rice in Africa (Carney, 1998; Nayar, 2010). According to Nayar (2010), many rice varieties from India were introduced in East Africa during the historical period of Asian rice introduction in Africa. Our results, taken together with the history of rice cultivation in Africa and Asia, suggest that African rice blast may have originated from India or China. South East Asia has previously been identified as the centre of origin of rice blast (Saleh *et al.,* 2014) . Moreover, a worldwide analysis of genotypes based on the *ACE* gene confirmed long distance migration of rice blast (Tharreau *et al.*, 2009). In some instances it was shown that one population is more closely related to a population from other continents than from a population from the same area. According to the authors, long distance migration is possible through contaminated seed.

Our results indicate that some genes involved in secondary metabolism of *M. oryzae* are conserved in SSA isolates compared to Guy11. Some of the genes are involved in a range of biochemical processes that are important for survival for the pathogen in stressful or toxic environments. For example, stress-activated map kinase-interacting proteins are important in stress signalling therefore enabling the pathogen develop a rapid and robust stress responses (Smith *et al.,* 2010). Nucleotidyl transferase are important in DNA repair and replication

(Aravind & Koonin, 1999) whereas aldehyde dehydrogenases are involved in metabolism of toxic aldehydes, accumulated under stress conditions (e.g. osmotic and heat shock, glucose exhaustion) to less reactive forms (Navarro-Aviño *et al.,* 1999). This indicates that the genes may be essential in conferring useful traits to enable the SSA isolates adapt to the harsh hot, dry and sunny conditions experienced in the tropical regions. Future studies may help us gain insights on whether the genes are involved in pathogenesis under stressful environmental conditions.

Effectors function by suppressing host immunity (Hogenhout et al., 2009), modulating metabolism (Djamei *et al.*, 2011) and preventing recognition of the invading pathogen (Mentlak *et al*., 2012) thereby allowing the pathogen to colonise the host. The interaction between *M. oryzae* and the host conforms to the longstanding gene-for-gene theory i.e. for every resistance gene (R) in the host there is a corresponding avirulence gene (*AVR*) in the pathogen, as formulated by Flor (1971) and described for *M. oryzae* by Silué *et al.* (1992). The AVR genes encode for proteins that are recognized by genotypes of the host plant harbouring a cognate resistance gene. This leads to effector-triggered immunity, often accompanied by a hypersensitive reaction in which the invaded cells die thereby limiting spread of the pathogen to other cells. Our results indicate that the candidate effector gene did not complement Guy11. The complemented isolates were compatible with monogenic lines harbouring R genes that are cognate to the targeted *AVRs*. The candidate effector gene was highly expressed during infection of infection of KE0002 on rice blast susceptible variety, Mokoto, indicating that the gene plays a role in the plant infection process. Based on our studies we cannot conclusively determine if the candidate gene is an effector or a different virulence factor. There is a need to undertake more studies to clarify

role of the candidate gene in rice infection process. There is also a need to undertake studies to determine localisation of the gene product in rice cells and determine if it is consistent with previously reported localisation patterns of known effectors. We also need to disrupt the function of the gene in KE0002 by targeted gene deletion and conduct infection assays. This will complement our infection assay results and rule out ambiguities that may arise due to hypersensitive reaction from the complemented strains. In addition, we need to determine the interaction between the candidate gene product and the host plant resistance proteins by undertaking yeast-two hybrid and co-immuno precipitation studies.

# 6 Chapter 6. General Discussion

In this study, I have demonstrated regional differences in the genotypic diversity of rice blast isolates from Kenya. Genotypic analysis of Kenyan isolates based on two markers, ITS and DNA fingerprinting with Pot2 element, showed that rice blast isolates from Central Kenya are distinct from those from Western and Coastal Kenya. These results were validated by more robust genome sequence analysis. A similar pattern was also observed in virulence diversity of the isolates. The genotypic diversity may be due to differences in rice varieties grown in these regions which impose specific selection pressure on the rice blast

However, there is an information gap in understanding the R and QTL repertoire of rice germplasm in Kenya. Our results indicate that nucleotide variations in the coding region of *AVR-PITA* may have resulted in loss of function in some of the Kenyan isolates. In order to fully understand host-pathogen interaction in the Kenyan rice blast population, there is a need to screen Kenyan rice germplasm for rice blast resistance genes (R) and QTL. Our study findings show that IR-2780-1, NERICA 1, NERICA 2, NERICA 4, NERICA 10, NERICA 11 are resistant to rice blast. These varieties may be harbouring R and QTLs that are effective on Kenyan populations of *M. oryzae.*

Most R and QTLs have been identified using the classical QTL mapping/linkage analysis from F2 generation, double haploid (DH), back cross (BC), recombinant inbred lines (RILs) or near isogenic lines (NILs) populations and includes the following steps (i) crosses are undertaken from two selected parents with trait(s) of interest. This results into a segregating mapping populations; (ii) construction of linkage map using suitable markers; (iii) measurement of phenotypic traits from 50-250 progenies under diverse environmental conditions; (iv) selection of polymorphic markers in parents and the progenies; (v) the markers are ordered

and statistically correlated with phenotypic traits in the mapping populations in order to discover QTL. This process is time consuming and its long before new QTLs are identified (Shabir *et al.,* 2017). Advancements in technology has made it possible to hasten the process by utilising genotyping-by sequencing (GBS) to develop numerous SNPS between parents and progenies and correlating the SNPS to phenotypic data. Genome wide association mapping involves scanning the whole genome in order to identify genomic regions with traits of interest on all the chromosomes and includes the following: (i) selection of diverse germplasm from natural populations; (ii) Measuring trait of interest; (iii) Genotyping of mapping populations; (iv) determine linking disequilibrium (LD) and population structure; (v) use statistical methods to correlate phenotype and genotype data.

Rice is a good candidate for GBS and genome-wide mapping because its genome is relatively small and has been sequenced. GBS has been utilised to identify QTLs associated with rice blast. Recently Mgonja *et al.* (2016) undertook GBS on 162 rice diversity panel 1 (RDP1) (70 indica and 92 temperate japonica) in order to determine genomic regions associated with rice blast resistance against eight isolates from four African countries. Association mapping showed that 31 regions were associated with rice blast resistance. About 1/3 of the *RABRs* had LOD scores > 5.0 and their effects on resistance to *M. oryzae* were as strong as those of R genes. Conversely, the remaining 2/3 had low LOD scores and were considered to be QTLs. The study identified a major rice blast resistance genomic region, RABR*_2,* which is linked to *Pish* R gene on chromosome 1. In a similar study, Mgonja *et al.* (2017) undertook GBS and rice blast resistance association mapping of 190 African cultivars and identified 23 genomic regions associated with rice blast resistance (RABRs). *RABR*_23 was associated with *Pita* R gene on chromosome 12. Taken together, these results

demonstrate that GWAS is a robust tool for identification of QTLs and R genes in rice. GWAS is performed on a diversity panel in order to maximise on diversity of alleles and haplotypes. Rice diversity panel 1 (RDP1) is a collection of 421 purified homozygous lines, representing a broad range of genetic variation within *O. sativa* and is widely used for GWAS studies (Eizenga *et al.*, 2014). Whereas this is advantageous in identifying new QTLs, it requires that identified QTLs must be validated in a breeding population before they are utilised in marker assisted selection (MAS). It is therefore advantageous to perform GWAS on adapted breeding lines, as exemplified by Begum *et al.* (2015). QTLs identified in this manner can directly be used in marker assisted breeding and favourable and unfavourable haplotypes can be identified from the segregating breeding lines thereby ensuring efficient selection of breeding parents. MAS of favourable haplotypes increases breeding efficiency and reduces cost by reducing the number of plants advanced to the next generation. In the study, Begum *et al.* (2015), performed association mapping for 19 agronomic traits including yield, and its yield components, in a breeding population of elite tropical irrigated breeding lines. Using GBS, 71,710 SNPs were identified and GWAS performed with the goal of hastening the breeding program. Using this breeding panel the authors identified 52 QTLs for 11 agronomic traits, including large effect QTLs for flowering time and grain length/grain width/grain-length-breadth ratio.

A similar strategy can be utilised to develop durable rice blast breeding in SSA Africa. For example, in Kenya our study has identified elite cultivars that showed high levels of tolerance to rice blast. In addition, there is a large pool of local landraces that have relatively long standing cultivation by farmers in Coastal Kenya. Although these landraces may harbour desirable traits, their genetic architecture is unknown and, consequently, the genetic pool they represent has

not been exploited in breeding programs. Future studies should therefore focus on characterising the lines and identifying novel sources of rice blast resistance genes that can be utilised in rice blast breeding programs.

During association mapping, progeny with desired traits are identified by exposing the germplasm to diverse environmental conditions. In association mapping for rice blast resistance, it is important that the germplasm is exposed to the full spectrum of virulence in the population. Understanding the population structure of *M. oryzae* is therefore a prerequisite for rice blast association mapping. Our study has revealed the population structure of *M. oryzae* in Kenya and established a biobank of rice blast isolates. Furthermore, we have sequenced selected isolates making it possible to study host-pathogen interactions in future studies. The information and resources arising from this study are therefore important tools for scientific community involved in developing durable rice blast resistance and are expected to be a pivotal resource in combating rice blast in SSA.

To develop durable resistance to rice blast in Africa, a breeding strategy must focus on introgressing both partial and complete resistance. Commercial varieties that harbour a combination of major R genes and QTLs are known to be more durably resistant to rice blast. For example, Moroberekan harbours two R genes (Pi5 and *Pi7*) and ten QTLs and has a longstanding resistance to rice blast (Wang *et al.,* 1994). Our study has identified *Piz5, Pita, Pita 2, Pi9* , *Piz* and *Pi12* rice blast resistance genes as suitable candidates that can be deployed in combination with QTLs such as *Pi35* and the recessive R gene *Pi21* (Zhou, 2017) to confer durable resistance to elite cultivars grown in Kenya. Breeding strategies must, however, also involve stakeholder and farmer participatory varietal selection to ensure that the developed cultivars are widely adopted by farmers. A

regional network of participatory varietal selection has successfully been used to develop farmer preferred varieties in 17 countries in West and Central Africa (Ones *et al*., 2000).

In conclusion, our study supports the hypothesis that the *M. oryzae* population outside the centre of origin comprises of distinct clonal lineages that have limited virulence spectrum. Moreover, the study has identified sources of resistance that can be utilised in breeding programs. Future studies should focus in monitoring the evolution of the pathogen and identifying new sources of resistance.

Appendix 1. List of *M. oryzae* isolates collected from rice growing regions in Kenya

| Isolate | Variety sampled | Part of Plant sampled | Location of Collection | GPS coordinates | Year Sampled |
|---|---|---|---|---|---|
| KE0001 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 38.23''/ E 37º 21' 39.68'' | 2013 |
| KE0002 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 38.23''/ E 37º 21' 39.68'' | 2013 |
| KE0003 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 38.23''/ E 37º 21' 39.68'' | 2013 |
| KE0005 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0006 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0007 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0008 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0009 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 25.20''/ E 37º 23' 41.20' | 2013 |
| KE0010 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0011 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0012 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0013 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0014 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0015 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |
| KE0016 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09''/ E 37º 21' 46.27'' | 2013 |

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|---|---|---|---|---|---|
| KE0017 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0019 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0020 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0021 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0022 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0023 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0024 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0025 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 43.09"/ E 37º 21' 46.27" | 2013 |
| KE0026 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 18.70", E 37º 22' 40.32" | 2013 |
| KE0027 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 15.71"/ E 37º 22' 39.32" | 2013 |
| KE0028 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 15.71"/ E 37º 22' 39.32" | 2013 |
| KE0029 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 44' 15.71"/ E 37º 22' 39.32" | 2013 |
| KE0030 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 59.50"/E 37º 22' 49.36" | 2013 |
| KE0031 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 59.50"/E 37º 22' 49.36" | 2013 |
| KE0032 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0034 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0035 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |

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| KE0036 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0037 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0038 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0040 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE0041 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2013 |
| KE 0200 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.50"/E 37º 22' 44.36" | 2014 |
| KE 0201 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0202 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0203 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0204 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0205 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0206 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0207 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0208 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 24.43"/E 34º 56' 14.61" | 2014 |
| KE 0209 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.88"/E 37º 22' 44.50" | 2014 |
| KE 0210 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |
| KE 0211 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |

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| KE 0212 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0213 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.88"/E 37º 22' 44.50" | 2014 |
| KE 0214 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |
| KE 0215 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.51"/E 37º 22' 42.29" | 2014 |
| KE 0216 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |
| KE 0217 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |
| KE 0218 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.56"/E 37º 22' 43.74" | 2014 |
| KE 0219 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |
| KE 0220 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.45"/E 34º 56' 14.80" | 2014 |
| KE 0221 | Basmati 370 | Neck | Western Kenya, Ahero | S 0º 38' 57.51"/E 37º 22' 44.29" | 2014 |
| KE 0222 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0223 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0224 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0225 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.51"/E 37º 22' 44.29" | 2014 |
| KE 0226 | ITA 310 | Leaf | Western Kenya, Ahero | Sº 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0227 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.51"/E 37º 22' 44.29" | 2014 |
| KE 0228 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 44.77"/E 34º 56' 18.46" | 2014 |

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| KE 0229 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 44.77"/E 34º 56' 18.46" | 2014 |
| KE 0230 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.88"/E 37º 22' 44.50" | 2014 |
| KE 0231 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.68"/ E 37º 22' 44.47" | 2014 |
| KE 0232 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.68"/ E 37º 22' 44.47" | 2014 |
| KE 0233 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0234 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0235 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.88"/E 37º 22' 44.50" | 2014 |
| KE 0236 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.68"/ E 37º 22' 44.47" | 2014 |
| KE 0237 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.68"/ E 37º 22' 44.47" | 2014 |
| KE 0238 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.64"/ E 37º 22' 44.53" | 2014 |
| KE 0239 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0240 | Basmati 370 | Neck | Central Kenya, Wanguru | S 0º 38' 57.64"/ E 37º 22' 44.53" | 2014 |
| KE 0241 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 38.85"/E 34º 56' 18.77" | 2014 |
| KE 0242 | Basmati 370 | Collar | Central Kenya, Wanguru | S 0º 38' 57.64"/ E 37º 22' 44.53" | 2014 |
| KE 0243 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0244 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0245 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |

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| KE 0246 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0247 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0248 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0249 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0250 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0251 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0252 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0253 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0254 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0255 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0256 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0257 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0258 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0259 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0260 | ITA 310 | Leaf | Western Kenya, Ahero | S 0º 8' 25.71"/ E34º 56' 18.37" | 2014 |
| KE 0300 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28' 34.27"/E 34º 57' 20.05" | 2014 |
| KE 0301 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28' 34.27"/E 34º 57' 20.05" | 2014 |

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| KE 0302 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28' 34.27"/E 34º 57' 20.05" | 2014 |
| KE0303 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0304 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0305 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0306 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0307 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34$^{0}$ 32’ 43.25’’ | 2014 |
| KE0308 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0309 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0310 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0311 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0312 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0313 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0314 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0315 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0316 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0317 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34 32’ 53.10’’ | 2014 |
| KE0318 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |

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| KE0319 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0320 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0321 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0322 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.23’’/E 34º 32’ 53.10’’ | 2014 |
| KE0323 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0327 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0328 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0329 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE 0330 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0331 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0332 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0333 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0334 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0335 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0336 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0337 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0338 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |

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| KE 0339 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 21014 |
| KE0340 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0341 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0342 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0343 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0344 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0345 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0346 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0347 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0348 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0352 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0353 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0354 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0355 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0356 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 25.58’’/ E 34º 32’ 53.03’’ | 2014 |
| KE0357 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0358 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |

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| KE0359 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0360 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.38’’/E 34º 32’ 52.30’’ | 2014 |
| KE0361 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0362 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0363 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0364 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0365 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0366 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0367 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0368 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0369 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0370 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0371 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0372 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0373 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0374 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0375 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |

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| KE0376 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0377 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0378 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0379 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0380 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0381 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0382 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0383 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0384 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0385 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0386 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0387 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0388 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0389 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0390 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0391 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0392 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |

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| KE0393 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0394 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0395 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0396 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0397 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0398 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0399 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0400 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 26.89’’/E 34º 32’ 52.52’’ | 2014 |
| KE0401 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0402 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 33.45’’/E 34º 32’ 43.25’’ | 2014 |
| KE0403 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0404 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0405 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0406 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0407 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0408 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0409 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |

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| KE0410 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0411 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0412 | Saro | Leaf | Western Kenya, Homa-Bay | S 0º 28’ 24.79’’/E 34º 32’ 53.14’’ | 2014 |
| KE0413 | Saro | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0414 | Luyin | Leaf | Coastal Kenya, Kikoneni | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0415 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0416 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 2.27'/E 39º 7'' 29.77' | 2014 |
| KE0417 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 2.27'/E 39º 7'' 29.77' | 2014 |
| KE0418 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 32'' 58.21'/E 39º 7'' 44.43' | 2014 |
| KE0419 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE 0420 | Nerica 19 | Leaf | Coastal Kenya, Kikoneni | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE 0421 | Mbuyu | Leaf | Coastal Kenya, Kikoneni | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE 0422 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0423 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE 0424 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33'' 1.94'/E 39º 7'' 30.48' | 2014 |
| KE0425 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 28' 20.07''/E 39º 19' 55.04'' | 2014 |
| KE0426 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 28' 20.07''/E 39º 19' 55.04'' | 2014 |

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| KE0427 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 28' 20.07"/E 39º 19' 55.04" | 2014 |
| KE0428 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 28' 20.07"/E 39º 19' 55.04" | 2014 |
| KE0429 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0430 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 28' 20.07"/E 39º 19' 55.04" | |
| KE 0431 | Mbuyu | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33" 1.94'/E 39º 7" 30.48' | 2014 |
| KE0432 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 28' 20.07"/E 39º 19' 55.04" | |
| KE0433 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 32' 58.21"/E 39º 7' 44.43" | 2014 |
| KE 0434 | Luyin | Leaf | Coastal Kenya, Kikoneni | S 4º 28' 20.07"/E 39º 19' 55.04" | 2014 |
| KE0435 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 2.33"/E 39º 7' 29.80" | 2014 |
| KE0436- | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0437 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0438 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 2.33"/E 39º 7' 29.80" | 2014 |
| KE0439 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 2.33"/E 39º 7' 29.80" | 2014 |
| KE 0440 | Niwahi | Leaf | Coastal Kenya, Kikoneni | S 4º 33" 1.94'/E 39º 7" 30.48' | 2014 |
| KE0441 | Supa | Leaf | Coastal Coastal Kenya, Lunga Lunga Kenya, Lunga Lunga | S 4º 32' 56.98"/E39º 7' 43.33" | 2014 |
| KE 0442 | Supa | Leaf | Coastal Kenya, Kikoneni | S 4º 33" 1.94'/E 39º 7" 30.48' | 2014 |

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| KE0444 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 2.33"/E 39º 7' 29.80" | 2014 |
| KE0445 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 2.33"/E 39º 7' 29.80" | 2014 |
| KE 0446 | Supa | Leaf | Coastal Kenya, Kikoneni | S 4º 33" 1.94'/E 39º 7" 30.48' | 2014 |
| KE0447 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 3.33"/E 39º 7' 30.80" | 2014 |
| KE0448 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.35"/E 39º 7' 40.80" | 2014 |
| KE0449 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.35"/E 39º 7' 40.80" | 2014 |
| KE0450 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.35"/E 39º 7' 40.80" | 2014 |
| KE0451 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0452 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39$^{0}$ 7' 20.80" | 2014 |
| KE0453 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0454 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0455 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 2.33"/E 39º 7' 29.80" | 2014 |
| KE0456 | Niwahi | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0457 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 6.33"/E 39º 7' 35.80" | 2014 |
| KE0458 | Luyin | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0459 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0460 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33" 1.94'/E 39º 7" 30.48' | 2014 |

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| KE0461 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0462 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 6.33"/E 39º 7' 35.80" | 2014 |
| KE0463 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 6.33"/E 39º 7' 35.80" | 2014 |
| KE0464 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0465 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0466 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0467 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0468 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5.33"/E 39º 7' 36.80" | 2014 |
| KE0469 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 4.33"/E 39º 7' 35.80" | 2014 |
| KE0470 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 6.33"/E 39º 7' 35.80" | 2014 |
| KE0471 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0472 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0473 | Mbuyu | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0474 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5"/E 39º 7' 20.80" | 2014 |
| KE0475 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5.33"/E 39º 7' 35.80" | 2014 |
| KE0476 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 4.33"/E 39º 7' 35.80" | 2014 |
| KE0477 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 6.33"/E 39º 7' 35.80" | 2014 |

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| KE0478 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0479 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0480 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 5.33"/E 39º 7' 35.80" | 2014 |
| KE0481 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0482 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0483 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0484 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0485 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0486 | Mbuyu | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0487 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0488 | Niwahi | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0489 | Mbuyu | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0490 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0491 | Luyin | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0492 | Mbuyu | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |
| KE0493 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 1.94"/E 39º 7' 30.48" | 2014 |
| KE0494 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |

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| KE0495 | Supa | Leaf | Coastal Kenya, Lunga Lunga | S 4º 33' 29.47"/E 39º 6' 57.89" | 2014 |

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